UNIVERSITÉ DE TOURS

ÉCOLE DOCTORALE : SSBCV

UMR CNRS 7261, Institut de Recherche sur la Biologie de l'Insecte

THÈSE présentée par :

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Soutenue le : **14 mars 2024**

pour obtenir le grade de : **Docteur de l'université de Tours**
Discipline/ Spécialité : Sciences de la vie et de la santé

LA COGNITION DES LARVES DE MOUSTIQUE COMME INDICATEUR BIOLOGIQUE DE LA QUALITE DES ECOSYSTEMES AQUATIQUES

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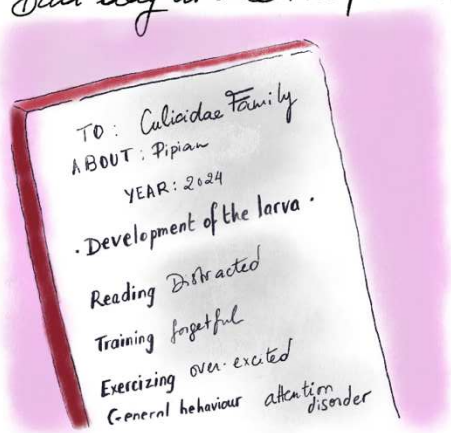
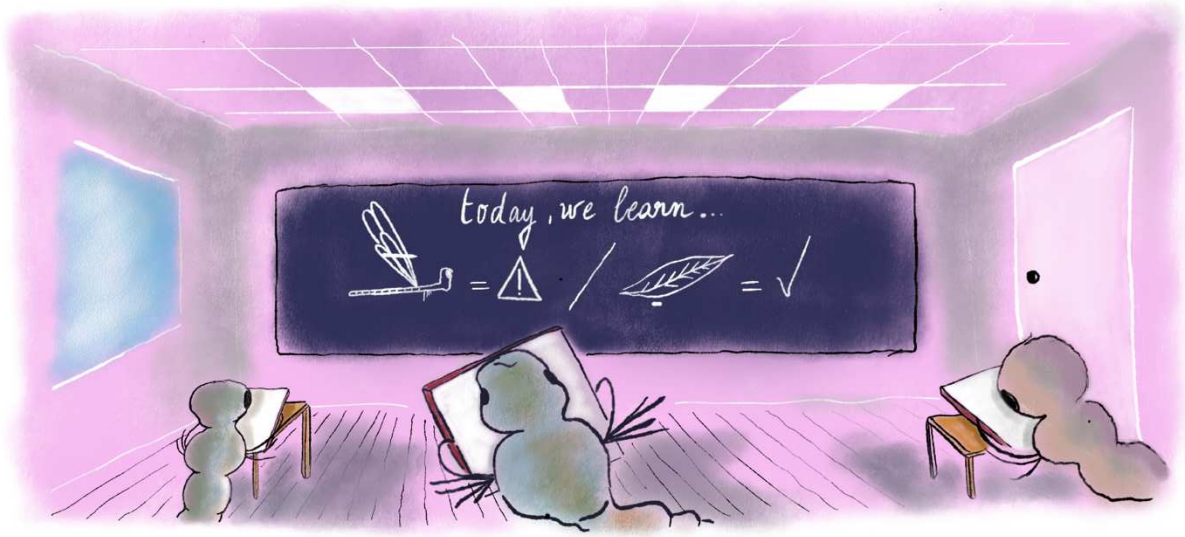
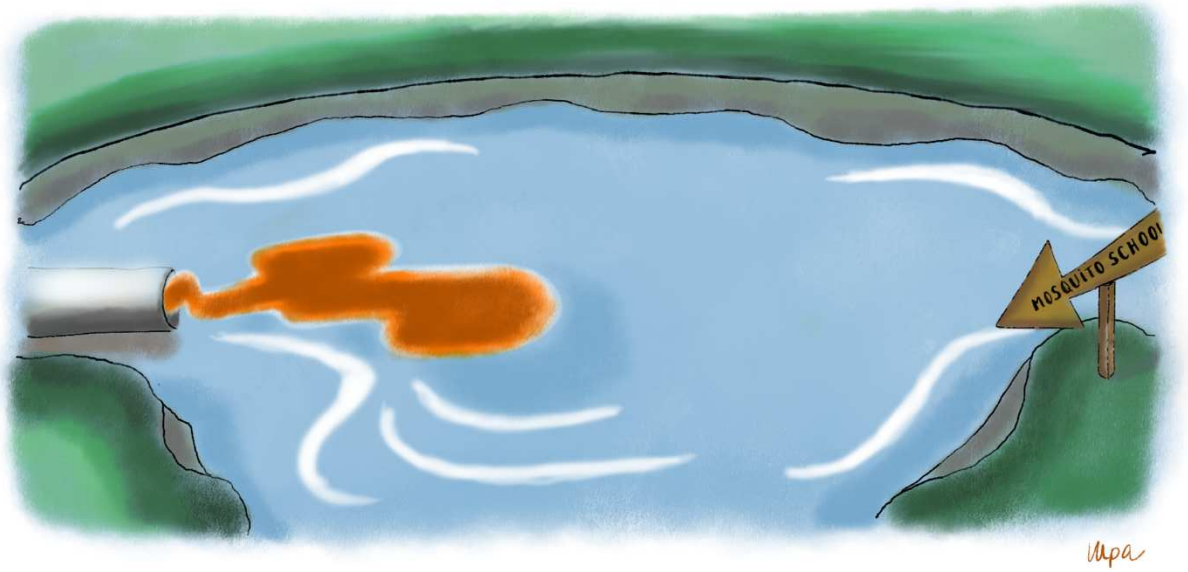
Bad day at the Mosquito School







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Remerciements

La réalisation d'une thèse est une aventure périlleuse. Tous les jours, nous sommes dans la quête de la vérité et tous les jours, nous sommes confrontés à notre propre ignorance. N'ayant pas fait de licence de biologie, j'ai particulièrement eu ce syndrome de l'imposteur pour compagnon de route. Cependant, je dois en premier lieu remercier mes encadrants qui m'ont grandement aidé à affronter ce quotidien, qui ont entretenu ma curiosité, m'ont permis de m'assumer, gagner en maturité et dépasser les blocages rencontrés durant cette thèse.

Claudio, je suis extrêmement reconnaissant du temps que tu m'as accordé. Que ce soit pour me rassurer sur ma présentation en visio depuis un autre pays ou me répondre en une dizaine de secondes pour me donner une piste de réflexion, tu as toujours été très disponible. Chaque relation doctorant-encadrant est unique et tu as su nous donner des orientations claires et nous faire adopter les règles qui nous ont permis de mener à bien ce projet. Merci pour ta bienveillance, merci pour ta patience, merci pour tes explications passionnantes et tous nos échanges inspirants. J'espère m'être procuré un peu de ta rigueur scientifique, et avoir compris l'intérêt de la biologie expérimentale, ainsi que le raisonnement, la démarche et le langage qu'elle impose. Je commence à comprendre l'importance de « l'effet Lazzari » et je ressens beaucoup de gratitude pour la confiance que tu m'as accordée et pour le climat encourageant et motivant que tu as su mettre en place.

Fernando, merci d'avoir reconnu mes compétences et de m'avoir donné l'opportunité de travailler sur ce projet. Merci pour ta présence pendant ces trois années et merci d'avoir veillé à ce que les conditions matérielles et financières soient réunies pour le bon déroulement de ma thèse. Grâce à toi, j'ai pu m'affirmer dans mon travail, développer une pensée critique, faire face à l'incertitude et trouver les ressources nécessaires pour surmonter les difficultés créées durant ces trois ans.

Je remercie l'ensemble de mon jury de thèse pour avoir accepté d'évaluer mon travail, et en particulier les rapporteurs pour leur travail de relecture. Je remercie également les membres de mon comité de thèse, effectué à mi-parcours : **David Baracchi, David Carrasco**, **Joël Meunier** et **Martin Giurfa**.

Je remercie l'école doctorale SSBCV, et en particulier **Joëlle Dupont** et **Florian Guillou** pour leur suivi attentionné durant ces trois années. Je remercie les membres du projet COMPORTATE, **Nathalie Gassama**, **Renaud Baeta**, **Arnaud Leroy**, **Laetitia Fougère**, **Emilie Destandeau** et **Ali Fadel** pour les échanges que nous avons eu.

L'IRBI est un laboratoire peuplé de bestioles bizarres, enfermées dans des salles d'élevage, que l'on essaie de comprendre avec difficulté, et qui font de la recherche. Ce sont surtout des créatures fascinantes que je remercie, et avec qui j'ai eu l'occasion de travailler, discuter, ou jouer aux fléchettes. Je remercie **David Giron**, directeur de l'IRBI, pour son accueil et sa bienveillance. Je remercie très chaleureusement **Sylvain** et **Christelle** pour leur accompagnement pendant ces trois années. Merci de votre écoute, de votre disponibilité, de votre soutien et de votre sérénité contagieuse. Je remercie sincèrement **Joël**, mon parrain, pour son inspiration, son réalisme et sa bienveillance. Merci d'avoir trouvé les mots quand j'en avais besoin, merci d'avoir cru en moi et merci de m'avoir aidé à croire en moi. Je remercie tous les membres de l'équipe INOV pour l'atmosphère de bienveillance et de pertinence scientifique qu'ils dégagent. En particulier, merci à **Miguel** et **Nicolas** pour leur aide dans le développement du système de tracking.

Je remercie les personnes qui ont partagé mon bureau. **Mourad**, petit ange parti trop tôt, merci pour ta sérénité et nos discussions. **Gwen**, ma phase analyseur te remercie pour toute la motivation et l'inspiration que tu dégages. **Thomas**, merci pour ces quasi 30 ans de blagues, tu fais partie du top 3 de mes voisins de bureau préférés de l'IRBI. Vous allez me manquer !

Je remercie amicalement tous les doctorants et non permanents que j'ai croisé durant ces trois années et avec qui j'ai partagé des moments sérieux, notamment des parties de fléchettes, de ping pong, billard, des sorties escalades, des sorties guinguettes, des soirées aux misérables, des escape game, des week-ends cohésion et autres work'chopes. **Béa**, **Matthieu**, **Marina**, **Alexandra C**, **Robert**, **Loretta**, **Lucas**, merci de m'avoir accueilli au début de mon parcours et de m'avoir aussi bien intégré à l'IRBI. **PAM, Jeremy**, merci pour ces moments sport à l'escalade, que ce soit en salle, en falaise ou vachés aux misérables. **Mathieu L**, **Louis**, **Mélissa**, **Paul**, **Solène**, **Marina**, **Eva**, **MC**, **Violette**, **Harmony**, **Simon**, **Nachida**, **Marina**, **Martin C**, merci de m'avoir écouté dire des bêtises tous les midis et d'avoir tenté (avec peu de succès) de me battre aux fléchettes. Merci pour votre enthousiasme au quotidien, et pour avoir, sauf banni, respecté les règles des devinettes. Spéciale dédicace à : **Alexandra T**, you gave me some very important russian links, loved it ; **Mathieu B**, pour m'avoir guidé dans ma quête de meilleur dresseur ; **Laura**, **Camille** et **Corentin** pour notre organisation des Olympiades ; **Romain H**, **Florian**, merci pour vos vrais et vos faux conseils, vos encouragements, vos blagues nulles et surtout nos réunions quotidiennes à 16h en salle de pause ; **Fred**, je ne sais pas quand on aura l'occasion de faire d'autres soirées mais imagine.

Je remercie sincèrement **Simon**, **Aurélie**, **Aicha**, **Carole L**, **Laureen**, **Karine** et **Esteban** pour leur sympathie, leur aide et leur bonne humeur quotidienne. Merci à **Marlène**, **Marie** et **Samuel** de m'avoir permis d'enseigner dans d'excellentes conditions. **Elfie**, merci pour toutes nos discussions et nos encouragements mutuels. Je remercie aussi les personnes avec qui j'ai beaucoup apprécié échanger et qui ont été d'excellent conseil : **Christophe**, **Florent**, **Jérôme**, **Charlotte**, **Elisabeth**, **Romain L**.

Je remercie **Carole D** pour son incroyable accompagnement. Nos échanges m'ont permis d'avancer sereinement. Je remercie **Alexia**, **Solène** et **Didier** pour être restés de grands enfants. Je remercie **Rachel** ainsi que **Muriel** et sa famille. J'ai une pensée affectueuse pour **Maeliss**, **Laura**, **Livie**, **Agathe** et **Leo-Paul** qui ont apporté beaucoup de sens à mon quotidien à Tours. **Ludo**, je chérie notre amitié, nos envolées lyriques, nos entraînements de capoiera et nos balades en canoë. **Clément**, je suis ravi d'avoir partagé des explorations de grotte, des bidouillages électroniques, des matchs de Béhourd, des fêtes de la science, des spectacles, des concerts, des débats et des soirées interminables. Je remercie mes colocs d'amour, **Marion**, **Agnès**, **Thorsen**, **Arthur** et **Julie** pour tout le bonheur que vous m'avez apporté au quotidien, pour toutes les discussions, repas et bêtises que nous avons partagés (sauf Julie).

Will et **Magali**, je suis redevable de vos conseils, du soutien et de l'inspiration que vous m'apportez depuis déjà huit ans.

Je remercie également mes amis **Basti-Bastou**, **Laulie**, **Félix Féraille**, **Pierrot**, **Felckin**, **Boulon**, **Vicente** et **Val** pour l'amour que nous nous portons, pour le bonheur de nous retrouver à chaque fois, pour l'inspiration créatrice et pour l'énergie que nous partageons. Je vous aime fort. Clin d'œil à mon maitre spirituel d'escalade **Tom**.

Je remercie **JB**, **Guigui**, **Dan**, **Max**, **Adri,** la famille **Aillères**, la famille **Becanne**, la famille **Dessart**, ma belle-sœur **Inès**, ma mère **Myriam**, mon frère **Maxime**, ma nièce **Jana**. Vous êtes pleinement responsables de ma détermination, de ma connerie et de ma joie de vivre.

Il est bien connu que les gens ne lisent que les remerciements. Je profite donc de l'attention de ceux qui comptent s'arrêter là pour les inviter à aller voir rapidement les beaux résumés graphiques des trois premiers chapitres[1]. Ci-dessous figure le résumé, la question à laquelle il correspond, et la page associée. Bonne lecture.

M

Est-ce que les larves de moustique peuvent apprendre ? Réponse page 84

Combien de temps peuvent-elles se souvenir ? Réponse page 114

Est-ce que des polluants peuvent affecter leur comportement ? Réponse page 141

[1] Idée piquée du manuscrit d'Antoine Cribellier.

Préambule

Cette thèse a été effectuée de février 2021 à janvier 2024 au sein de **l'Institut de Recherche sur la Biologie de l'Insecte** à Tours. Elle a été encadrée par l'école doctorale 549 - Santé, Sciences Biologiques et Chimie du Vivant (SSBCV) et financée par le projet **APR IR 2020 COMPORTATE** de la Région Centre Val de Loire. L'accès aux étangs a été facilité par la Direction de l'Attractivité des Territoires du Département d'Indre-et-Loire.

Notre travail a donné lieu à **quatre publications** (deux publiées, une en cours de soumission, une en préparation), à l'encadrement d'un stagiaire de Master 1ère année et à **huit communications orales** (six nationales et 2 internationales). Durant ces trois ans, j'ai également eu l'opportunité de donner 70,5 heures d'enseignements en 2021 et 2022, et la chance de valoriser mon stage de Master 2ème année, qui a donné lieu à une publication et à trois communications orales.

Liste des publications incluses dans le manuscrit :

Dessart M, Piñeirúa M, Lazzari C, Guerrieri F (2023) Assessing learning in mosquito larvae using video-tracking. Journal of Insect Physiology 149:104535. https://doi.org/10.1016/j.jinsphys.2023.104535

Dessart M, Lazzari C, Guerrieri F (2024) Habituation leads to short but not long term memory formation in mosquito larvae. Journal of Insect Physiology. 155:104650. https://doi.org/10.1016/j.jinsphys.2024.104650

Dessart M, Lazzari C, Guerrieri F (2024) Acute and chronic sublethal chemical pollution affects activity, learning and memory in mosquito larvae. Manuscript submitted

Dessart M, Lazzari C, Guerrieri F (2024) Environmental conditions affect activity and cognition of mosquito larvae, but not responsiveness to visual stimuli. Manuscript in preparation

Liste des publications non incluses dans le manuscrit :

Dessart M, Aguiar JMR, Tabacchi E, Guillerme S, Giurfa M (2024) Color-Advertising Strategies of Invasive Plants through the Bee Eye. Frontiers in Plant Science. https://doi.org/10.3389/fpls.2024.1393204

Liste des communications orales :

Dessart M, Tabacchi E, Aguiar JM, Guillerme S, Giurfa M. Invasive plant species through the bee eye: an analysis of flower coloration in the French Pyrenees – **Poster presentation** - *51ème Colloque de la Société Française pour l'Etude du Comportement Animal* - France | Mai 2021

Dessart M, Tabacchi E, Aguiar JM, Guillerme S, Giurfa M. Invasive plant species through the bee eye: an analysis of flower coloration in the French Pyrenees – **Invited speaker** - *University of São Paulo* – São Paulo, Brazil | Mai 2021

Dessart M, Piñeirúa M, Lazzari C, Guerrieri F. The influence of physico-chemical characteristics of aquatic ecosystems on prey- predator interactions. A behavioural-neuroanatomical approach – **Invited speaker** - *IRBI UMR 7261* - Tours, France | Oct 2021

Dessart M, Tabacchi E, Aguiar JM, Guillerme S, Giurfa M. Invasive plant species through the bee eye: an analysis of flower coloration in the French Pyrenees - **Invited speaker** - *Département d'Ecologie, Physiologie et Ethologie UMR 7178* - Strasbourg, France | Oct 2021

Dessart M, Piñeirúa M, Lazzari C, Guerrieri F. Visual learning abilities in mosquito larvae are not impaired neither by light deprivation nor by turbidity – **Oral presentation** - *European PhD Network "Insect Science" - XII Annual Meeting* – Florence, Italie | Nov 2021

Dessart M, Piñeirúa M, Lazzari C, Guerrieri F. A tracking system to assess activity and learning in mosquito larvae – **Poster Presentation** - *Ecology & Behaviour Meetings – 15th Edition* – Strasbourg, France | Mar 2022

Dessart M, Lazzari C, Guerrieri F. Effect of two herbicides and one medical drug on locomotor activity and learning in Aedes aegypti – **Oral presentation** – *34ème Colloque Biotechnocentre* – Centre Val de Loire, France | Oct 2022

Dessart M, Lazzari C, Guerrieri F. Chemical pollution affects the behaviour of mosquito larvae – **Poster presentation** - *52ème Colloque de la Société Française pour l'Etude du Comportement Animal* – Tours, France | Mai 2023

Dessart M, Lazzari C, Guerrieri F. Chemical pollution affects the behaviour of mosquito larvae – **Oral presentation** – *DiPEE Centre d'Etudes Biologiques de Chizé* - Chizé, France | Juin 2023

Dessart M, Lazzari C, Guerrieri F. Cognitive abilities in mosquito larvae as indicators of environmental quality – **Oral presentation** – *Behaviour 2023* – Bielefeld, Allemagne | Août 2023

Dessart M, Lazzari C, Guerrieri F. The cognition of mosquito larvae as a biological indicator of environmental quality – **Oral presentation** – *Rencontre annuelle du Club de Neuroéthologie – Société des Neurosciences* – Albi, France | Mai 2024

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Chapitre 1 : Introduction

Je fais commerce d'amitié avec l'araignée.

Jean Henri **Fabre** (1879) Souvenirs entomologiques

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La place des écosystèmes aquatiques dans un monde anthropisé

Les écosystèmes aquatiques…

De toutes les ressources que la Terre nous offre, l'eau est sans doute la plus particulière, la plus importante, et la plus en danger. Élément clé de la vie et du maintien des structures cellulaires, ses propriétés chimiques de solvant lui permettent de dissoudre d'autres éléments à l'échelle atomique. Ses propriétés physiques thermodynamiques lui permettent de transférer de l'énergie, et l'eau permet également le transport de nutriments, par exemple dans le sang ou la sève. Les cycles de l'eau façonnent les paysages et régulent les processus biologiques complexes (Singh et al. 2020).

Sur Terre, 97,3% de l'eau mondiale est salée. Dans les 2,7% restants, environ 68% de **l'eau douce** est sous forme de glace et de glaciers, 30% restant se situe dans les nappes phréatiques et seulement 1,3% comprend les lacs et les rivières, qui représentent donc 0,035% de l'eau liquide sur terre (Madhav et al. 2020). Pourtant, cette faible surface terrestre présente la plus **forte densité d'espèces**, et environ **10% de la biodiversité totale** (Strayer et Dudgeon 2010 ; Figure 1). Cette biodiversité comprend de nombreux taxa, qu'ils soient vertébrés chez les poissons et amphibiens, invertébrés chez les crustacés, les mollusques, les insectes ou chez les plantes (aquatiques, algues) (Winemiller 2018).

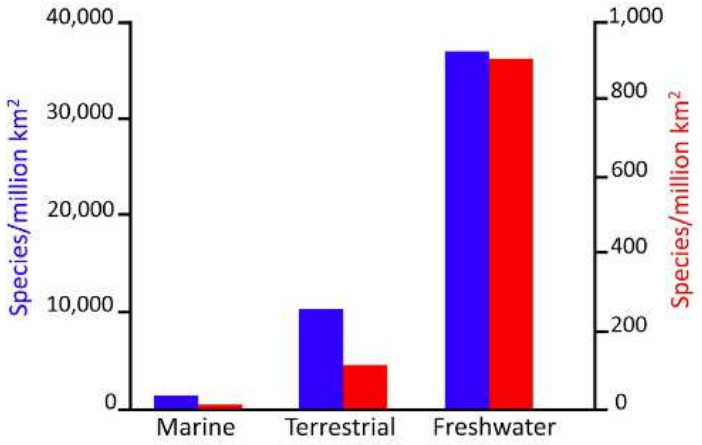


Figure 1: Densité d'espèces décrites (en bleu) et en danger (en rouge) chez les eucaryotes dans les écosystèmes marins, terrestres et d'eau douce. D'après Winemiller (2018).

… comme source de biodiversité et de service écosystémique

Par ailleurs, l'eau douce est extrêmement utile et est au cœur d'actions à différentes échelles. D'un point de vue industriel, l'eau douce est majoritairement puisée dans les nappes phréatiques et permet de réguler des processus de fabrication notamment en refroidissant des équipements, ce qui représente 15% de son exploitation, ou 770 km^3/an. L'eau douce est également critique au secteur agricole en jouant un rôle fondamental dans l'irrigation, la préparation des sols, la protection contre le gel et la production de culture, comptant pour environ 76% de son exploitation, soit 3810 km^3/an. Enfin, l'eau douce est essentielle à la santé des habitants et représente 9% de son utilisation, ou 380 km^3/an (Carpenter et al. 2011).

En dehors de l'importance de l'eau *per se,* les écosystèmes d'eau douce répondent également à de nombreux **services écosystémiques** (Hanna et al. 2017 ; Figure 2). La valeur monétaire des services écosystémiques fournit par les écosystèmes d'eau douce a été évaluée à 6,5 billions de dollars par an, correspondant à 20% de la valeur totale des services écosystémiques provenant de la Terre (Strayer et Dudgeon 2010). Concernant les utilisations ci-dessus, ces services comprennent l'approvisionnement en eau et l'irrigation agricole. Ils sont également liés à la production d'énergie hydraulique, comprennent plusieurs rôles socio-économiques (commerce,

tourisme, transport, sports aquatiques), et sont également liés à l'alimentation (pêche, élevage et transformation d'espèces aquatiques en nourriture). Enfin, ils permettent de maintenir la biodiversité, possèdent un rôle important de filtration des contaminants et d'assainissement des eaux, ils recyclent de la matière organique, piègent du carbone et peuvent agir comme tampon naturel contre les crues (Vári et al. 2022).

Néanmoins, malgré cette importance économique, sociale et culturelle, une troisième particularité de ces écosystèmes d'eau douce provient de leur état de santé. En effet, ces écosystèmes sont **les plus dégradés et les plus en danger dans le monde**, notamment via six menaces : altérations hydrologiques, dégradation et perte de l'habitat, pollution, surexploitation, introduction d'espèces invasives et changements climatiques (Arthington 2021). Un tiers des espèces vivant dans les écosystèmes d'eau douce sont considérés par l'IUCN comme en danger d'extinction (IUCN, 2021) et entre 10 000 et 20 000 espèces sont probablement disparues à cause des activités humaines (Strayer 2006). Plus d'un tiers des surfaces d'eau douce a été réduite entre 1970 et 2015, et un tiers seulement des rivières s'écoule sans retenue de leur source à la mer (Lynch et al. 2023).

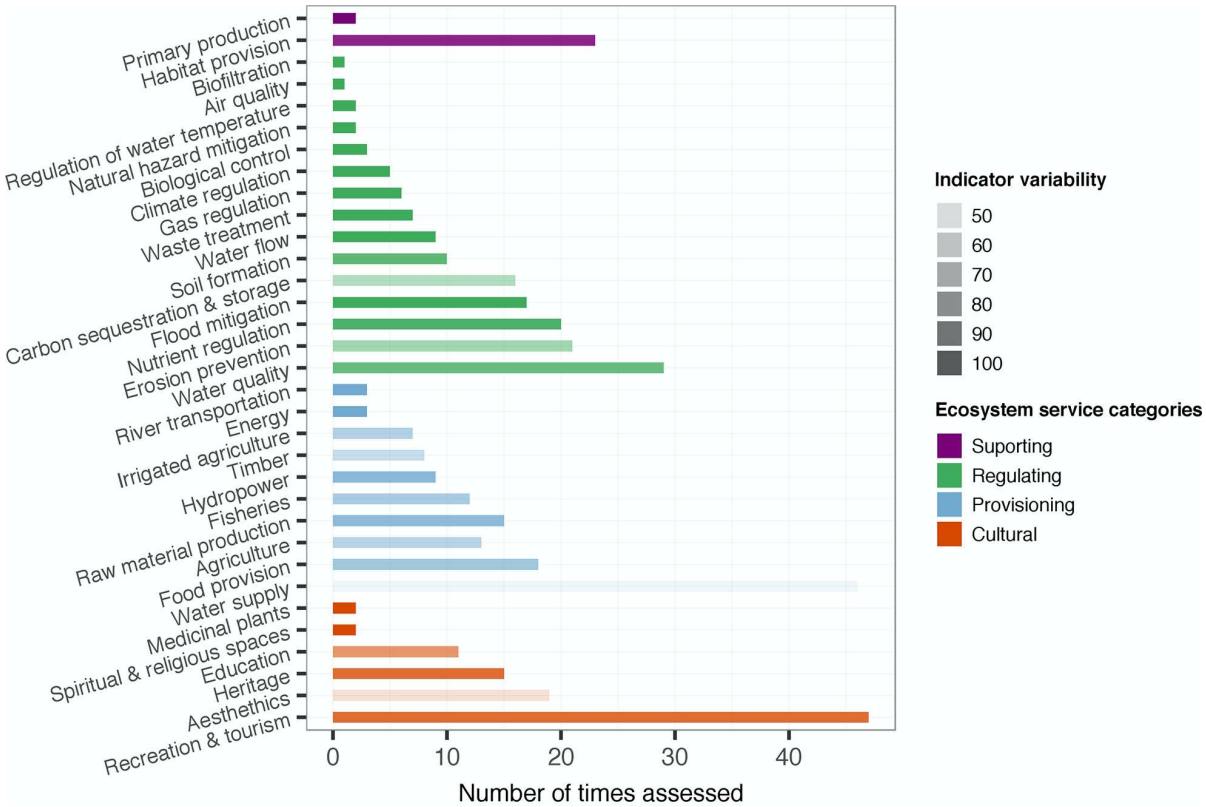


Figure 2: Variabilité des indicateurs et des services écosystémiques. L'indicateur de variabilité représente la diversité du nombre de variables utilisées pour décrire chaque service écosystémique. Plus la couleur est claire, plus l'indicateur est consistant. Plus la barre est longue, plus le service est évalué. D'après Hanna et al. (2017).

Dégradation des écosystèmes : implications pour la biodiversité

Ces dégradations proviennent majoritairement de **l'activité anthropique**. En effet, l'explosion de la **démographie** et de la dispersion humaine du siècle dernier a augmenté la surface des sols exploités (Oberdorff 2022). Ces deux facteurs ont accompagné le besoin d'augmenter rapidement les rendements agricoles, qui, avec l'essor des technologies, en particulier lié à **l'agriculture**, ont favorisé la fragmentation des territoires et l'usage de **substances chimiques**, herbicides, insecticides, rodenticides et fongicides (Goel et Aggarwal 2007; Savci 2012; Gill et al. 2018). De ces activités et de leur impact dépendent grandement des économies, avec notamment des pays présentant les plus forts taux de biodiversité dans les écosystèmes d'eau douce qui sont aussi les lieux avec les plus forts taux de pauvreté (Lynch et al. 2023). Les pays en développement sont également ceux où l'on manque le plus de données concernant l'état de ces écosystèmes et enfin les lieux où l'eau est la plus susceptible d'être contaminée (Singh et al. 2020; Faghihinia et al. 2021).

Enfin, **en France**, le constat est mitigé. La grande diversité des écosystèmes de ce pays habite une faune et une flore diversifiées qui suscitent le développement de plusieurs programmes de **restauration des cours d'eau,** de surveillance de la qualité de l'eau et de gestion durable des ressources en eau (Souchon et Keith 2001; Brown et al. 2009). De plus, plusieurs actions ont été menées afin de préserver des écosystèmes et notamment les zones humides. Un exemple fort provient de la Zone A Défendre de Notre Dame des Landes, expérimentation sociale qui a pour ambition de préserver une zone humide (Renard 2013). Cependant, les écosystèmes d'eau douce en France sont également soumis aux mêmes menaces, en particulier l'introduction d'espèces invasives notamment via de nombreux cours d'eau majeurs et de ports à travers le pays (Devin et al. 2005). Les rivières sont dégradées par la destruction et la dégradation des

habitats naturels, et en particulier par l'introduction de substances chimiques (Bayramoglu et al. 2020).

Ce travail de thèse s'intéresse à l'évaluation des écosystèmes d'eau douce. Ces derniers représentent une ressource clé pour les humains. Leur exploitation intensive et leur dégradation nuisent fortement aux services qu'ils peuvent fournir ainsi qu'à la riche biodiversité qui les comprend. En particulier, l'introduction de polluants en lien avec l'activité humaine et agricole perturbe fortement les interactions au sein de ces écosystèmes.

Écotoxicologie : évaluer l'effet de polluants sur les écosystèmes

Effets des polluants : état des connaissances

Un **polluant** est un agent physique, chimique ou biologique, introduit par les activités anthropiques, qui provoque des effets indésirables dans un environnement naturel (Elliott 2003). Les principales sources de polluants sont les métaux lourds, les engrais (principalement azote, phosphore, potassium), les huiles/graisses/hydrocarbures, les micro/nano-plastiques, les déchets solides/sédiments en suspension, la pollution thermale et radioactive, les micro-organismes, les **composés pharmaceutiques** et **les pesticides** (Boyd 2010; Madhav et al. 2020; Singh et al. 2020; Speight 2020).

Lorsque l'on veut mesurer ou représenter l'effet de polluants sur le vivant, il est nécessaire de prendre en compte **la durée de l'exposition** de ce polluant. Les études distinguent généralement deux types d'exposition, aigüe et chronique, dont les durées sont empiriques (Smital 2008). La **toxicité aigüe** correspond à une exposition ponctuelle voire unique, à un polluant à haute dose. Les effets sont mesurés rapidement après exposition. A l'inverse, la **toxicité chronique** réfère à une exposition à long terme, répétée, à un polluant à dose basse ou moyenne. Les effets se développent donc ici sur une période prolongée (Vestel et al. 2016). Ces deux toxicités bénéficient de modèles

conceptuels différents (ex : Reichwaldt et al. 2016) car elles peuvent agir sur des voies physiologiques différentes chez les organismes cibles (Weltje et al. 2013).

Comment fait-on pour polluer les écosystèmes d'eau douce ?

Bien que les quantités et l'intensité des applications de polluants définissent en grande partie leur toxicité dans les écosystèmes d'eau douce, il est également nécessaire de prendre en compte leurs **sources**, **voies d'accès** et leur **persistance** dans les systèmes hydrologiques (Figure 3, Gilliom 2006). Concernant les sources, deux types sont recensées : sources ponctuelles et non ponctuelles (aussi nommées sources diffuses, Speight 2020). Les premières correspondent à des sources pouvant être identifiées, quantifiées et réglementées, comme les effluents d'eaux usées municipales et industrielles. En revanche, les sources non ponctuelles sont diffuses et difficilement mesurables, comme le ruissellement des terres agricoles ou les précipitations (Carpenter et al. 2011).

Dans un souci de concision, le champ d'application de ce travail se limite au rôle des pesticides et des composés pharmaceutiques sur les écosystèmes d'eau douce, illustré dans le **Chapitre 4**. En l'occurrence, ce travail de thèse s'inscrit dans le projet *COMPORTATE*, financé par la région Centre-Val de Loire. Celle-ci présente une grande diversité de milieux aquatiques naturels et artificiels. Le projet *COMPORTATE* a pour but de caractériser l'évolution de l'état de certains écosystèmes d'eau douce sélectionnés dans la région. Les écosystèmes sélectionnés sont présentés dans le **Chapitre 5**.

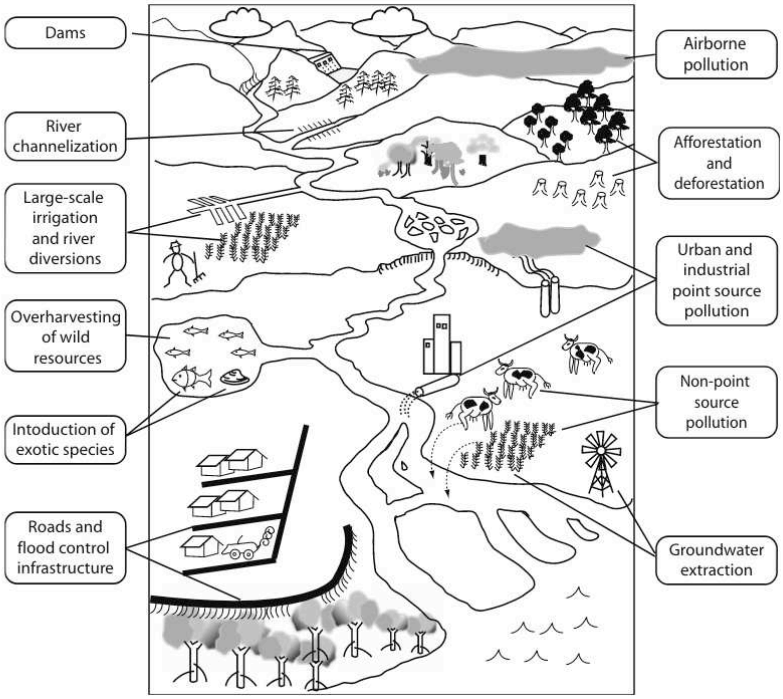


Figure 3: Exemples de voies d'accès susceptibles de polluer les écosystèmes aquatiques. Barrages hydrauliques, modifications hydrologiques, irrigation, élevage, espèces invasives, route, barrages anti-inondation, déforestation, sources ponctuelles urbaines et industrielles, sources diffuses, extraction des eaux souterraines. D'après Carpenter et Biggs (2009).

Effets des pesticides en mono ou co-exposition

Un **pesticide** est une substance utilisée pour la lutte contre les insectes, mauvaises herbes, champignons, rongeurs ou micro-organismes. Les pesticides agissent en contrôlant et en tuant leurs espèces cibles. Ils présentent de nombreux avantages, comme l'augmentation du rendement agricole ou la réduction des maladies transmises notamment par les insectes, mais leur utilisation soulève beaucoup de problématiques autour de leur **sélectivité**, c'est-à-dire de leur impact sur l'organisme cible et sur l'environnement où vit cet organisme (Goel et Aggarwal 2007). Tous les pesticides sont composés d'une **substance active**, celle qui va agir sur l'organisme ciblé, et une majorité contiennent une gamme d'adjuvants qui vont agir sur les propriétés de la substance active pour améliorer son transport et son efficacité (Gilliom 2006). La nature et l'ampleur de l'utilisation des pesticides n'ont cessé d'évoluer depuis les années 1980, avec une application d'environ 2,5 millions de tonnes de pesticide chaque année, ce nombre étant en augmentation. De plus, moins de 0,1% des pesticides utilisés

atteint réellement leur organisme cible, et les 99,9% restants se dispersent dans l'air, le sol et l'eau, entraînant la pollution des écosystèmes (Pimentel 1995). Par ailleurs, de nouvelles substances actives sont introduites chaque année et des milliers de noms de produits différents sont commercialisés, chacun contenant un ou plusieurs pesticides qui vont présenter des modes d'action différents (Gilliom 2006).

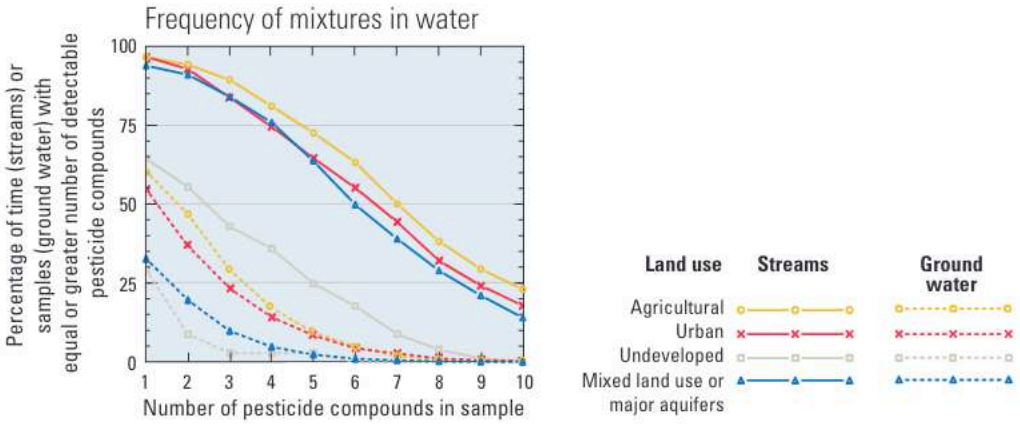


Figure 4: Cocktail de pesticides analysés dans les rivières de surface (lignes continues) et souterraines (lignes pointillées) en fonction du nombre de pesticides présents, en fonction du type d'habitat : agricole (jaune), urbain (rouge), non-urbanisé (gris), habitat mixte (bleu). D'après Gilliom (2006).

Dans les écosystèmes, les polluants vont donc plus souvent se retrouver de manière simultanée, que ce soit sous forme de substance active ou d'un produit de dégradation (Gilliom 2006; Figure 4). Ces substances chimiques sont susceptibles d'interagir entre-elles et l'ensemble de ces substances se qualifie de **cocktail** (Vasquez et al. 2014). L'évaluation des effets potentiels des pesticides doit donc prendre en compte les mélanges entre pesticides, les cocktails, mais aussi avec les autres types de polluants cités ci-dessus. Lorsque plusieurs composés sont en co-occurrence dans un écosystème, quatre types d'effets peuvent être rencontrés.

1) Lorsque les organismes cibles et les modes d'actions diffèrent, les pesticides peuvent ne pas interagir et être **indépendants** : chaque pesticide a l'effet qu'il aurait s'il était seul.

2) Lorsque deux pesticides ont un mode d'action et des structures chimiques similaires, l'effet peut être **additif**, c'est-à-dire qu'il correspond à l'addition de chaque effet individuel, normalisé aux doses de toxicités présentes.

3) A l'inverse, l'effet peut être **antagoniste** si les deux pesticides ont un effet moindre ensemble que séparément.

4) Finalement, l'effet est qualifié de **synergique** lorsque la toxicité est supérieure au modèle additif (Siviter et al. 2021).

Effets des herbicides : état des connaissances

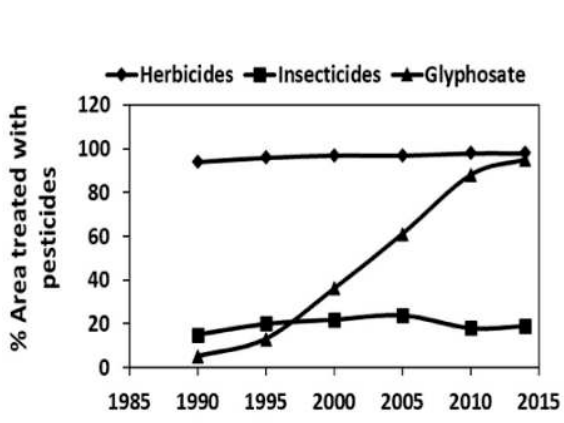
Les **herbicides** représentent une catégorie majeure de pesticide et sans doute la plus utilisée dans le monde (Sharma et al. 2018). Depuis les années 1940, ils sont massivement utilisés afin de réduire les mauvaises herbes en remplacement du travail mécanique du sol. Les herbicides varient selon leur mode d'absorption et leur persistance dans les sols, et peuvent être **sélectifs** ou non-sélectifs. Dans le premier cas, l'herbicide va cibler une plante via un mode d'action spécifique. Dans le second cas, l'herbicide va détruire toute la végétation sur des terres cultivées (champs en jachère), des terrains vagues ou des voies ferrées. L'augmentation de cultures transgéniques résistantes à un mode d'action (ex : maïs, soja) des herbicides a également favorisé le développement des herbicides non-sélectifs (Sharma et al. 2018).

Les herbicides ont permis d'accroître le rendement agricole et la rentabilité en limitant le labour et en conservant ainsi l'humidité des sols. De plus, les herbicides sont en expansion dans les pays du sud en étant bon marchés et en réduisant la main-d'œuvre (Gianessi 2013). Cependant, ils créent de nombreux risques éco-toxicologiques chez les organismes présents dans les écosystèmes à toutes les échelles trophiques. En effet, de nombreuses études mettent en évidence des **toxicités létales et sous-létales** de ces herbicides, notamment chez les invertébrés des sols et des écosystèmes aquatiques (Sharma et al. 2018).

Le **glyphosate**, *N*-(phosphonométhyl)glycine ($C_3H_8NO_5P$), est la substance active de l'herbicide le plus utilisé dans le monde. C'est un herbicide non-sélectif, foliaire et systémique, produit - de sa mise sur le marché en 1974 jusqu'en 2000 - exclusivement par la multinationale Monsanto (Giesy et al. 2000). Depuis 2000, il est entré dans le domaine public et est produit par d'autres entreprises. Le glyphosate est utilisé massivement et partout dans le monde, au tel point qu'en 2014, les agriculteurs avaient

pulvérisé suffisamment de glyphosate pour avoir appliqué en moyenne 0,5 kg/ha de glyphosate sur l'ensemble des terres cultivées du monde (Benbrook 2016; Figure 5). Le glyphosate (substance active) est souvent associé à différents adjuvants, notamment des surfactants afin d'augmenter sa capacité à se fixer et intégrer les cellules végétales et ainsi augmenter sa toxicité (van Bruggen et al. 2021). Le glyphosate a une demi-vie dans le sol de 2 à 215 jours et de 2 à 91 jours dans l'eau. Il est soluble dans l'eau, et lorsqu'il est utilisé aux doses recommandées, il ne s'accumule pas dans le réseau alimentaire (Gill et al. 2018). Cependant, le glyphosate se dégrade rapidement en AMPA qui se dégrade plus lentement et finalement en phosphate inorganique, en ammonium et en CO_2 (Battaglin et al. 2014).

Le glyphosate va agir au niveau foliaire et va se diffuser vers les racines (Gill et al. 2018). Il va agir en entravant la biosynthèse d'acides aminés aromatiques qui sont essentiels à la croissance de la plante. Spécifiquement, le glyphosate va entraver la production de l'enzyme 5-enolpyruvylshikimate-3-phosphate synthase de la voie métabolique du shikimate ou acide shikimique. Cette voie métabolique est présente chez les bactéries, mycètes, algues, protistes et Archaea mais pas chez les animaux. Auparavant, on pensait donc que le glyphosate ne nuisait pas aux organismes non ciblés présents dans l'écosystème, notamment par l'absence de cette voie métabolique (Gill et al. 2018). Cependant, de nombreuses études ont caractérisé la toxicité du glyphosate sur une large gamme d'organismes dans l'environnement, dans les sols et dans l'eau (pour des revues, voir : Giesy et al. 2000; Solomon et Thompson 2003; Battaglin et al. 2014; Gill et al. 2018; van Bruggen et al. 2021).



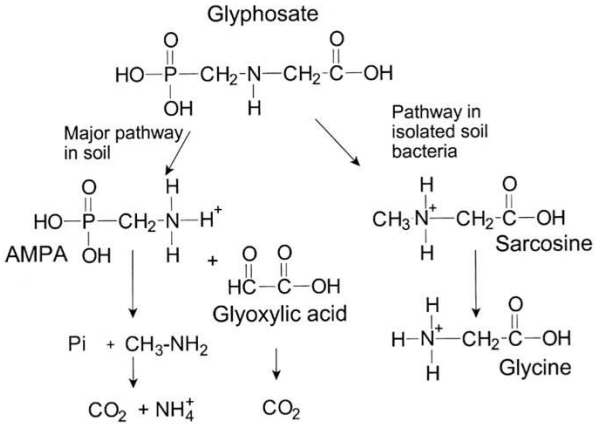


Figure 5: A) Augmentation de la surface de terres traitées au glyphosate, en comparaison avec la surface totale traitée aux herbicides et aux insecticides, aux USA entre 1990 et 2014. D'après van Bruggen et al. (2021). B) Voie métabolique de dégradation du glyphosate dans l'eau et le sol. D'après Solomon et Thompson (2003).

L'atrazine, 2-chloro-4-(éthylamine)-6-(isopropylamine)-s-triazine ($C_8H_{14}ClN_5$), est la substance active du second herbicide le plus utilisé dans le monde (Singh et al. 2018). Également systémique, mais sélectif, il a été introduit dans les années 1950 (Solomon et al. 1996). Très utilisé aux États-Unis, il est toutefois interdit dans l'Union européenne depuis 2003 du fait de la persistance dans les sols de ses métabolites pendant plusieurs années (Singh et al. 2018). Cet herbicide a une durée de demi-vie qui varie en fonction du pH, de la présence de bactéries ou de la température du sol, mais est globalement plus longue que le glyphosate (Rohr et McCoy 2010). Il se dégrade par des processus biologiques et chimiques, et ses métabolites varient en toxicité et en persistance (Graymore et al. 2001; Figure 6).

L'atrazine interfère avec les fonctions de la plante liée à la photosynthèse, d'une manière réversible si l'exposition est de courte durée (Solomon et al. 1996). L'atrazine est plus efficace dans les sols humides et est donc habituellement appliqué après les pluies, ce qui favorise son transfert dans les zones aquatiques, souterraines ou de surface (Graymore et al. 2001). Cet herbicide est également impliqué dans de nombreuses études de toxicité sur des organismes non-cibles (pour des revues voir : Solomon et al. 1996; Graymore et al. 2001; Rohr et McCoy 2010; Singh et al. 2018).

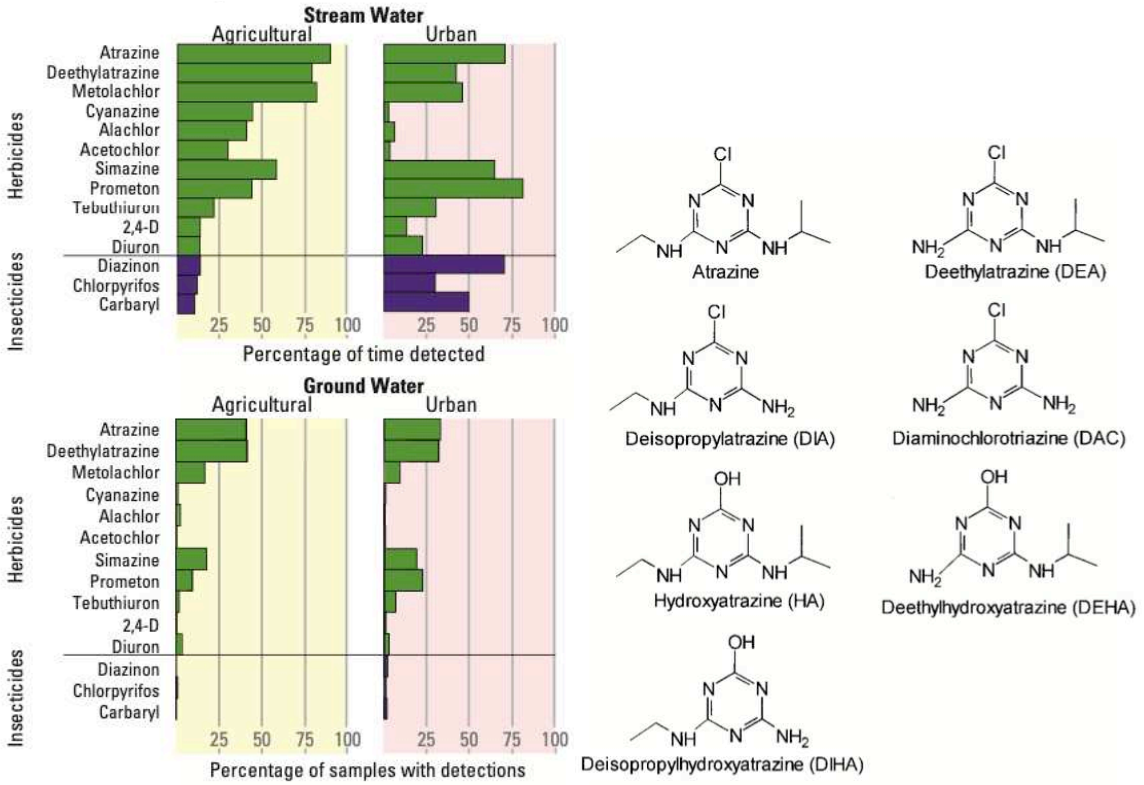


Figure 6: A) Pesticides les plus détectés dans les eaux de surface et souterraines aux USA entre 1992 et 2001 D'après Gilliom et al. (2001). B) Structure de l'atrazine et de ses métabolites. D'après Solomon et al. (1996).

Effets des produits pharmaceutiques : état des connaissances

Les **produits pharmaceutiques** ou **médicaments** sont des polluants dont l'usage est corrélé avec l'augmentation de la population et l'urbanisation depuis la seconde guerre mondiale (Thiebault et al. 2017a). Ces produits se retrouvent dans les écosystèmes après excrétion humaine ou animale et en aval de la phase de traitement dans les systèmes de gestion des eaux usées (Thiebault et al. 2017b). Environ 50% des médicaments sont prescrits, délivrés ou vendus de manière inappropriée, et 50% des patients ne les prennent pas correctement. (Fekadu et al. 2019). De nombreuses études ont recensées l'état des résidus pharmaceutiques dans les eaux et les sols, mais très peu de données existent sur le rôle éco-toxicologique de ces substances (Fent et al. 2006). Cependant, leur propriétés, notamment de résistance à l'inactivation, peuvent entrainer une bioaccumulation chez d'autres organismes et provoquer des effets toxiques dans les écosystèmes terrestres et aquatiques (Santos et al. 2010). Ils présentent des modes d'actions divers, mais sont néanmoins cités pour le

développement de bactéries et de gènes résistants aux antibiotiques ainsi que pour leur rôle de perturbateurs endocriniens (Danner et al. 2019).

Le **paracétamol** ($C_8H_9NO_2$) ou **acétaminophène**, est le médicament aux effets analgésiques et antipyrétiques le plus utilisé dans le monde (McCrae et al. 2018). Après avoir été mis en vente dans les années 1950, il a dépassé l'usage de l'aspirine dans les années 1980. Malgré son utilisation massive et sa vente libre dans la plupart des pays, son usage est toutefois remis en question ces dernières années vis à vis de potentiels effets néfastes sur les humains (McCrae et al. 2018; Nunes 2020). En France par exemple, c'est le médicament le plus prescrit avec 2,4 milliards de doses définies journalières, soit plus de 7,5 kilotonnes de paracétamol sur 3 ans ou une moyenne de 50 g de paracétamol par habitant et par an (Figure 7, Duong et al. 2016).

Chez les humains, le mode d'action du paracétamol reste méconnu, mais comprend un rôle au niveau du système nerveux central, similaire au rôle d'anti-inflammatoires non stéroïdiens. Il agirait par inhibition du site actif cyclooxygenase-2 qui a un rôle dans les processus de douleur et de fièvre (Duong et al. 2016). Dans les environnements aquatiques, le paracétamol est persistant et est fréquemment mesuré à de très fortes concentrations, notamment dans les pays émergents où le système de traitement des eaux usées n'est pas efficace (Fekadu et al. 2019). Dans ce cas, il peut agir sur les organismes selon plusieurs mécanismes distincts qui impliquent notamment le stress oxydatif et d'autres mécanismes antioxydants (Nunes 2020). Les études recensent des effets sur les plantes, polychètes, mollusques, crustacés et poissons, mais peu d'études écotoxicologiques existent (Santos et al. 2010; Nunes et al. 2014; Nunes 2020).

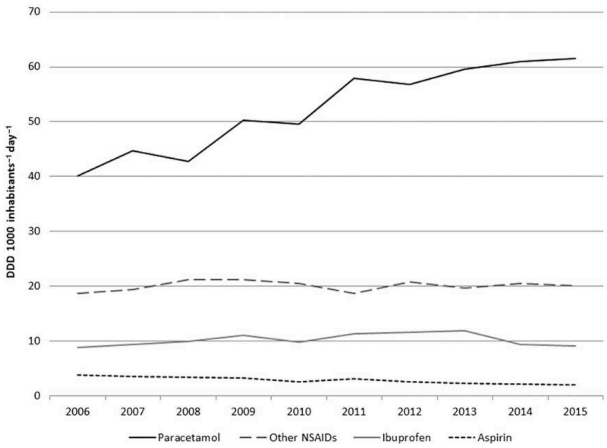


Figure 7: Consommation d'analgésiques non-opioïdes en France, entre 2006 et 2015. D'après Hider-Mlynarz et al. (2018).

Dans ce travail de thèse, j'ai mesuré l'effet de polluants, en particulier des herbicides et des médicaments, sur les organismes des écosystèmes d'eau douce. Ces effets sont complexes à appréhender. La co-occurence de plusieurs substances et leurs interactions, la difficulté de tracer les sources et les voies d'accès, les différents modes d'actions à différentes échelles de temps complexifient la mesure et la modélisation des effets potentiels de ces substances. Cependant, qu'il s'agisse des coûts économiques très importants liés au maintien des services écosystémiques, à la conservation de la biodiversité qu'ils contiennent, ou aux dommages croissants causés à ces écosystèmes, la gestion et les décisions gouvernementales associées aux écosystèmes d'eau douce doivent reposer sur des choix lucides, étayés par des mesures fiables et objectives. Derrière les constats préoccupants liés à l'évolution des usages et aux projections concernant les besoins en ressources et l'augmentation des contaminants, plusieurs types de méthodologies ont été développées afin de mesurer l'état de santé et la qualité des écosystèmes aquatiques d'eau douce.

Les indicateurs: entre variété et spécificité

Qu'est-ce qu'un indicateur ?

L'origine des premiers **indicateurs biologiques** d'écosystème est sans doute très ancienne. Aristote observait le comportement de poissons d'eau douce placés dans de l'eau salée ; les mineurs emportaient un canari dans les mines, qui indiquait un potentiel coup de grisou s'ils cessaient de chanter ou s'évanouissaient. Même si ces approches rudimentaires n'étaient pas exactement scientifiques, cette idée de s'inspirer du vivant pour tirer une conclusion de l'environnement est ancestrale et commune (Cairns et Pratt 1993).

Dès les années 1900, plusieurs méthodes émergent afin de mesurer l'état de santé ou la qualité des écosystèmes, notamment via la présence **d'invertébrés aquatiques**, présentés comme plus ou moins tolérants à la pollution (Hodkinson et Jackson 2005). Puis, à partir des années 1950, des indicateurs biologiques se développent à l'échelle mondiale basés sur la **présence** et **l'abondance** de certains organismes, au travers des programmes de surveillance de l'eau, et des organisations comme *l'Environmental Protection Agency* américaine (Barbour 1999). A partir des années 1980, les efforts se tournent vers l'utilisation d'outils en biologie moléculaire et en génétique afin d'évaluer la diversité génétique des populations ou la réponse à l'échelle moléculaire (Bolognesi et Cirillo 2014). Enfin, les nouvelles avancées modernes incluent des approches multimétriques et bioinformatiques, de modélisation, de séquençage haut débit, ainsi que l'utilisation de capteurs automatiques et de drones (Calvin Mordy et al. 2017; Lyons et al. 2019; Hentati-Sundberg et al. 2020).

Deux types de méthodologies existent afin de surveiller la qualité de l'eau et des écosystèmes aquatiques. La première est **directe** et consiste à prélever des **échantillons d'eau** et à évaluer la quantité et l'intensité de substances qu'elle contient. Si l'eau présente des concentrations élevées de substances considérées comme toxiques, l'eau est considérée comme contaminée. Cette méthodologie classique se base donc sur des **indicateurs chimiques** de l'eau (Madhav et al. 2020). La seconde méthode, **indirecte**, s'appuie sur la **présence ou l'état d'un organisme** qui va renseigner sur certaines caractéristiques écologiques de l'eau et des écosystèmes (paramètres abiotiques : physico-chimiques, microclimatiques, présence de polluants ; et paramètres biotiques : abondance, diversité, écologie fonctionnelle, etc) (López-

López et Sedeño-Díaz 2015). Les méthodes indirectes peuvent être séparées en deux catégories : les marqueurs biologiques, ou **biomarqueurs**, et les indicateurs biologiques, ou **bioindicateurs**. La première catégorie comprend les **mesures biochimiques** à l'échelle moléculaire, cellulaire ou tissulaire (Semren et al. 2018). La seconde correspond à une espèce, ou un **groupe d'espèces** dont la **présence**, **l'abondance**, la **physiologie** ou le **comportement** vont être utilisés pour évaluer la qualité d'un écosystème (Oertel et Salánki 2003).

Les enjeux liés à la mesure des écosystèmes aquatiques sont majeurs. En 2006, le budget mondial alloué à la gestion des ressources en eau dépasse les 100 milliards de dollars, illustrant l'importance de la santé des écosystèmes aquatiques et les enjeux liés au développement d'outils de surveillance de ces écosystèmes (Bonada et al. 2006).

Avantages et inconvénients

Concernant les **méthodes chimiques directes**, celles-ci permettent tout d'abord d'avoir une mesure quantitative. En effet, elles permettent de mesurer des quantités précises des propriétés organiques (quantité de nutriments, taux de O_2 dissous, etc) et de la présence de polluants. Ceux-ci sont également identifiables via des méthodes de chromatographies couplées à des méthodes de spectrométrie de masse (Bartram et al. 1996). Ces méthodes sont largement standardisées et permettent un suivi à long terme ainsi que des comparaisons au travers des territoires. En revanche, les méthodes chimiques directes sont coûteuses en argent et en temps. Elle nécessite des échantillonnages fréquents et du matériel sophistiqué. Ces méthodes qui correspondent à des photographies à un temps t ne prennent pas en compte les événements récents qui peuvent perturber la qualité d'un écosystème (conditions météo, saisonnalité ; Li et al. 2010). Si une molécule se dégrade vite dans l'eau, elle peut être présente dans l'écosystème mais être sous la limite de détection des appareils. La qualité des informations ne renseigne pas en elle-même la toxicité présente pour les organismes. Enfin, les analyses chimiques ne peuvent pas mesurer tous les composés présents, et ne peuvent détecter la présence de polluants que de manière unitaire, sans

prendre en compte les interactions additives, synergiques ou antagonistes (Oertel et Salánki 2003).

Les **biomarqueurs** présentent l'avantage d'être très précis concernant un changement subtil dans un écosystème (Oertel et Salánki 2003). Ils peuvent également intégrer des altérations à une échelle de temps plus longue qu'une mesure chimique, et peuvent répondre spécifiquement à une substance ou à un ensemble de substances précis (Bonada et al. 2006). A l'inverse, les biomarqueurs peuvent présenter des difficultés à relier une cause et sa conséquence sur la physiologie d'un organisme, un manque de standardisation des méthodes, et demandent souvent un coût élevé et une expertise approfondie (Azevedo et al. 2009; Zaghloul et al. 2020; Ford et al. 2021).

Enfin, les **bioindicateurs** présentent l'avantage principal d'intégrer l'effet cumulé de tous les composés présents afin de présenter une version plus globale de l'état de santé d'un écosystème (Hodkinson et Jackson 2005). En passant toute leur vie exposée aux conditions fluctuantes de ces écosystèmes, les bioindicateurs peuvent également permettre de mesurer une réponse dynamique à des changements ponctuels et de courtes durées, ou représenter une échelle de temps plus longue via l'accumulation d'effets délétères lorsqu'ils sont exposés à des polluants de manière chronique (Bonada et al. 2006). Les bioindicateurs permettent également de prendre en compte les interactions entre plusieurs facteurs (Oertel et Salánki 2003). Enfin, ils peuvent être utilisés à des échelles différentes : des communautés à l'espèce (López-López et Sedeño-Díaz 2015). Malgré ces avantages, les bioindicateurs peuvent entrainer des difficultés d'interprétation. En situation naturelle, les espèces sont affectées par plusieurs facteurs et par leurs interactions. Il peut donc être complexe de déterminer des relations de causalité. De plus, d'autres facteurs ne sont souvent pas pris en compte mais peuvent impacter les résultats, tels que les pathogènes, le parasitisme, la prédation, ou la compétition intra- et inter-spécifique (Zaghloul et al. 2020). Les bioindicateurs sont également limités par leur taxonomie. Par exemple, les insectes aquatiques ne peuvent pas servir à indiquer directement la réponse aux polluants de la biodiversité d'une autre classe. De plus, chaque effet mesuré à l'échelle de populations

ne pourra pas être inféré à l'échelle individuelle, sous peine de faire des raisonnements simplistes qualifiés d'« ecological fallacy » (Zaghloul et al. 2020).

Il n'y a pas de bon ou de mauvais indicateur - les critères

Le choix d'un indicateur chimique ou biologique va donc dépendre tout d'abord des questions que l'on se pose, puis de plusieurs facteurs comme ceux décrits ci-dessus. L'idéal serait de combiner plusieurs approches complémentaires à plusieurs échelles de temps et qui comprennent des analyses mécanistiques, mais cela nécessite un budget et une logistique conséquente (Oertel et Salánki 2003).

Par ailleurs, plusieurs auteurs proposent une liste de **critères** qui permettent de définir un bon indicateur. Hodkinson et Jackson (2005) proposent cinq critères :

1) Une facilité d'identification du taxon ;

2) Une bonne compréhension de la biologie de l'espèce et de sa réponse à des facteurs de stress / de changements d'habitats ;

3) L'espèce est abondante, facile à recenser et manipuler ;

4) Il y a une bonne adéquation entre l'espèce et les exigences spatiales et temporelles de l'étude ;

5) L'espèce ou le groupe d'espèce sont représentatifs de l'ensemble de la communauté, ou leurs réponses sont fortement corrélées avec un facteur de stress connu. Les auteurs précisent à la suite que le facteur esthétique et la signification économique de l'espèce doivent également être pris en compte, ainsi que les coûts et logistiques associés à son étude. Ces critères sont approfondis par Bonada et al. (2006) qui en proposent douze, répartis en trois catégories : justification, implémentation et performance. Ceux-ci correspondent en partie à ceux d'Hodkinson et Jackson en ajoutant la pertinence des indicateurs vis-à-vis des changements globaux et spécifiques des effets anthropiques (Bonada et al. 2006).

Hodkinson et Jackson illustrent la difficulté de développer un bioindicateur qui combine précision et faible coût. La détermination taxonomique, le choix des méthodes statistiques représentent également des challenges afin de développer un protocole de

suivi optimisé. Bonada et al. (2006) vont plus loin : ils comparent une ancienne et neuf nouvelles méthodologies aux critères qu'ils ont établis. L'approche « *Saprobian system* », très ancienne et très utilisée, ne remplit qu'un critère parmi douze, alors que les méthodes qui respectent le plus de critères n'en cochent que dix sur douze (Figure 8).

	Criterion number												
	Rationale					Implementation			Performance				
Biomonitoring approach	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**	**XII**	**Σ[b]**
Saprobian system	+	−	−	−	−	−	−	−	−	−	−	−	1
Biomarkers	−	−	−	±	+	±	+	+	?	?	?	±	6
Bioassays	+	±	±	?	+	±	+	+	±	?	±	±	10
Fluctuating asymmetry	+	±	−	+	±	±	+	+	?	−	−	?	7
Multimetric approaches	±	±	±	+	+	±	±	±	−	±	+	−	10
Multivariate approaches	+	±	?	+	±	+	+	±	−	±	?	±	9
Functional feeding groups	+	+	+	−	±	+	?	±	±	−	±	?	8
Multiple biological traits	+	+	+	+	+	+	+	+	+	+	?	?	10
Benthic secondary production	±	±	+	+	±	−	?	±	?	?	?	?	6
Leaf-litter decay	+	±	+	+	±	+	−	+	−	±	±	+	10

Figure 8: Résultats de l'analyse comparative de Bonada et al. (2006) de dix méthodes (une ancienne et neuf récentes) de bioindicateurs utilisant des invertébrés aquatiques. + : concordance avec le critère ; - : non concordance ; ± : concordance mitigée ; ?: non établi. b : somme des critères « + » ou «± ». Table adaptée de Bonada et al. (2006).

Les causes de cette inadéquation entre enjeux primordiaux de surveillance des écosystèmes d'eau douce et développement d'approches optimisées proviennent de plusieurs facteurs. En dépit de l'exigence des critères employés, les approches évaluées sont pour la plupart trop récentes pour montrer des résultats, en particulier sur le critère de performance. De plus, la finalité des indicateurs de surveillance est d'être transmis à des donneurs d'ordres et communiqué au grand public. Ce dialogue entre écologues de terrain ou biologistes expérimentaux, décisionnaires politiques et citoyens est difficile. D'une part, les données communiquées par les scientifiques sont trop complexes pour les non-scientifiques (voir par exemple le cas des réponses non-linéaires revu par Statzner et al. 1997). D'autre part, il est décourageant pour ceux qui développent des approches méthodologiques de voir qu'ils ne sont pas écoutés ou que leur approche est trop simplifiée (Bonada et al. 2006). Enfin, les personnes à même de prendre des

décisions sont contraintes par les politiques, lobbies, autorités, aux sources d'intérêts multiples (Statzner et al. 1997).

Un bon indicateur de la qualité des écosystèmes doit être adapté à plusieurs contraintes scientifiques, environnementales, logistiques, économiques et politiques. Pour l'instant, l'indicateur idéal n'existe pas. Sa recherche et son développement dépendent de variables financières, via le coût des méthodologies, logistiques, via la standardisation de méthodes déjà employées, humaines, par la communication entre scientifiques, décideurs et grand public. Les méthodes modernes bénéficient néanmoins du développement de plusieurs avancées technologiques et sont en cours de standardisation. Elles permettent d'explorer des questions plus précises en lien avec le contexte écologique. **Dans cette thèse, j'ai choisi de développer des expériences afin de tester la performance des insectes aquatiques comme indicateur biologique des écosystèmes d'eau douce.**

Utiliser les invertébrés aquatiques

Rôle des indicateurs biologiques pour évaluer la neuro-toxicité

Les méthodes d'estimation de la qualité d'un écosystème se développent à un rythme qui s'accélère. En effet, que ce soit par l'augmentation et la diversité des polluants de sources différentes dans les écosystèmes d'eau douce, par l'augmentation de la quantité de données disponibles ou par les besoins de prises de décisions, il y a une nécessité d'aller plus vite dans la surveillance des écosystèmes d'eau douce (Bertram et al. 2022; Wlodkowic et Jansen 2022). En parallèle, les effets observés de plusieurs polluants en fonction des concentrations ne correspondent pas toujours à des courbes monotones. En effet, les courbes dose-réponse peuvent être non-linéaires. Il est nécessaire de représenter des polluants à des doses proches de ce qu'il se passe en milieu naturel, c'est-à-dire à des doses réalistes (Hellou 2011). **Il faut donc aller plus vite mais aussi être plus précis**. Cependant, les doses de terrain produisent majoritairement des effets sous-létaux, c'est-à-dire que les polluants ne vont pas

directement provoquer la mort d'un individu mais vont le rendre déficient à répondre correctement vis-à-vis de son environnement. **Ainsi, pour mesurer ces effets sous-létaux, il faut aller au-delà des mesures d'abondance ou de diversité des espèces** (Chapman 2002)

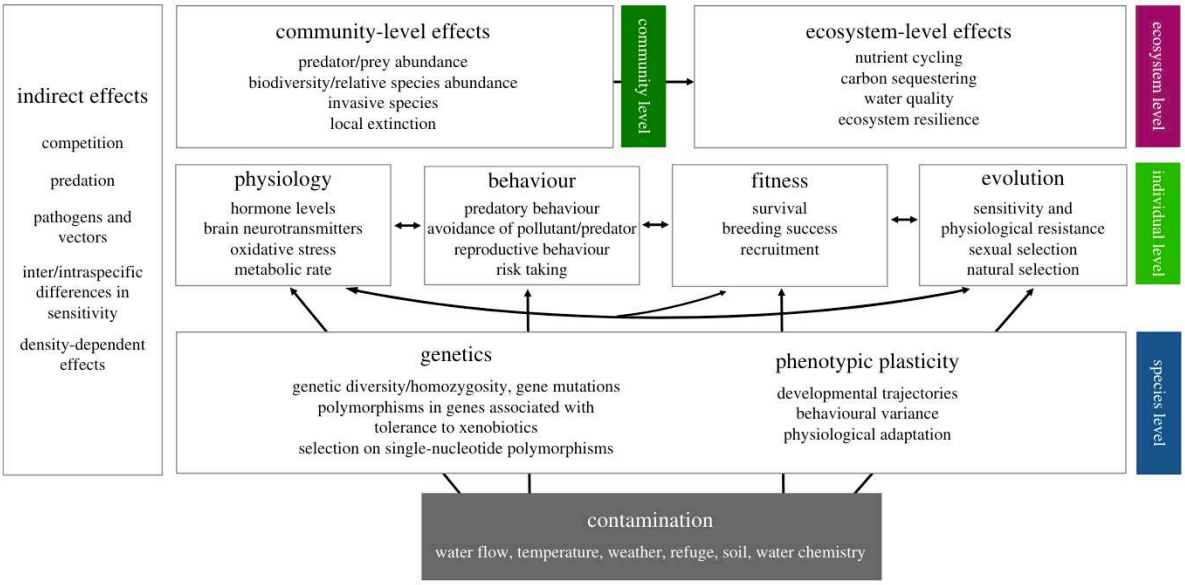


Figure 9: Cadre conceptuel développé par Saaristo et al. (2018) suggérant des approches méthodologiques pour identifier les effets de polluants sur le comportement animal. Pour chaque niveau d'organisation (individu en vert clair, espèce en bleu, communauté en vert foncé, écosystème en violet), plusieurs facteurs à quantifier ou à manipuler expérimentalement sont proposés. D'après Saaristo et al. (2018).

Que ce soit de manière aigüe ou chronique, les doses sous-létales vont principalement affecter le **système nerveux central** des animaux, c'est-à-dire avoir un effet neurotoxique. Les effets neurotoxiques de polluants ont des conséquences à court et à long terme sur les écosystèmes d'eau douce (Bownik et Wlodkowic 2021). Ils peuvent altérer les capacités de survie et de reproduction, modifier la durée de leur développement, provoquer des anomalies morphologiques. Les conséquences peuvent également cibler leur comportement : troubles moteurs, problèmes de coordination ; et la sensibilité aux stimuli : détecter un prédateur, trouver une source de nourriture ou s'orienter dans l'espace (Li et al. 2019; Andreazza et al. 2021; Fu et al. 2022). Ces altérations vont diminuer la fitness des espèces, et avoir un impact sur la capacité de l'écosystème à se maintenir et à assurer ses fonctions écosystémiques (Figure 9). Aujourd'hui, moins d'1% des composés chimiques vendus dans le monde ont été

évalués pour leurs propriétés neurotoxiques, et les protocoles en place sont insuffisants (Bownik et Wlodkowic 2021). Ainsi, plusieurs auteurs proposent de **lier ces protocoles d'écotoxicologie avec des approches d'écologie comportementale**. Ce pont est réalisable via l'utilisation des bioindicateurs et précisément via l'étude de leur comportement (Hicks et al. 2006; Hellou 2011; Ford et al. 2021; Bownik et Wlodkowic 2021; Bertram et al. 2022).

Aller plus loin grâce au comportement

Le **comportement** d'un animal constitue un « **endpoint** », c'est-à-dire une mesure, un paramètre qui va intégrer d'autres paramètres. En l'occurrence, le comportement est la manifestation physique la plus avancée et la plus fonctionnelle des fonctions physiologiques et neurologiques (Bownik et Wlodkowic 2021). En bref, même sans en être conscient, un animal va se comporter en fonction de ses capacités, ses besoins internes, et en réponse à son environnement. Lorsqu'un polluant va altérer les capacités d'un organisme, il est donc possible d'observer son comportement afin d'intégrer les possibles altérations de ce polluant.

Par exemple, une étude séduisante de Weidenmüller et al. (2022) a analysé l'effet du glyphosate sur le comportement de thermorégulation chez le bourdon *Bombus terrestris*. Les auteurs ont mis en évidence des effets à doses sous-létales du glyphosate chez les bourdons qui étaient nourris avec du sucre contenant du glyphosate : ceux-ci ont moins investi dans le comportement de thermorégulation. Mais en dehors d'une faible diminution individuelle dans la thermorégulation, c'est à l'échelle collective que l'effet de l'herbicide est fortement significatif : lorsque les colonies exposées au glyphosate ont expérimenté des limitations en ressources ainsi que des variations de températures, elles ont plus de difficultés à maintenir une température au-dessus de 28°C et la maintiennent moins longtemps que les colonies contrôles. Ce comportement de thermorégulation intègre ici des altérations neurologiques et/ou physiologiques et est quantifiable par une approche non-invasive. Cette étude montre également la nécessité de reproduire des conditions réalistes en mettant en évidence l'interaction entre évènements climatiques et exposition à un polluant.

Écotoxicologie comportementale chez les invertébrés aquatiques

Étudier le comportement d'organismes qui vivent dans des écosystèmes soumis à divers polluants permet donc de renseigner sur l'état de santé et les altérations de ces milieux. **L'écotoxicologie comportementale**, discipline qui a émergé dans les années 1960, est de plus en plus valorisée dans les études de toxicologie (voir les revues : Hellou 2011; Saaristo et al. 2018; Ford et al. 2021; Bownik et Wlodkowic 2021; Bertram et al. 2022). Parmi ces organismes étudiés, les **macroinvertébrés aquatiques** (taille supérieure à 0.5mm) présentent de multiples avantages. Ils sont principalement sédentaires et vont donc intimement refléter les conditions d'un milieu donné (Oertel et Salánki 2003). Leur biologie et taxonomie sont bien décrites. Ils peuvent représenter des micro-habitats en étant positionnés à différentes hauteurs le long de la colonne d'eau (Bonada et al. 2006). Ils ont des temps de développement très variables (quelques jours à plusieurs années), ce qui est utile pour renseigner des expositions à courte ou longue échelle (Ahmed 2023). Ils présentent un panel comportemental assez large et répondent fortement aux polluants, par comparaison aux plantes (Hodkinson et Jackson 2005). Ils sont compatibles avec des études en laboratoire à l'échelle individuelle et avec des logistiques simples et à bas coût (Oertel et Salánki 2003; Bonada et al. 2006). Enfin, comme illustré Figure 10, il existe aujourd'hui de nombreuses méthodes d'écotoxicologie qui incluent les invertébrés. Ces méthodes sont automatisées, de haut-débit et à faible coût. A noter que les insectes ne sont pas présents dans cette figure, alors qu'ils représentent 60% des espèces vivant dans les milieux aquatiques (Dijkstra et al. 2014).

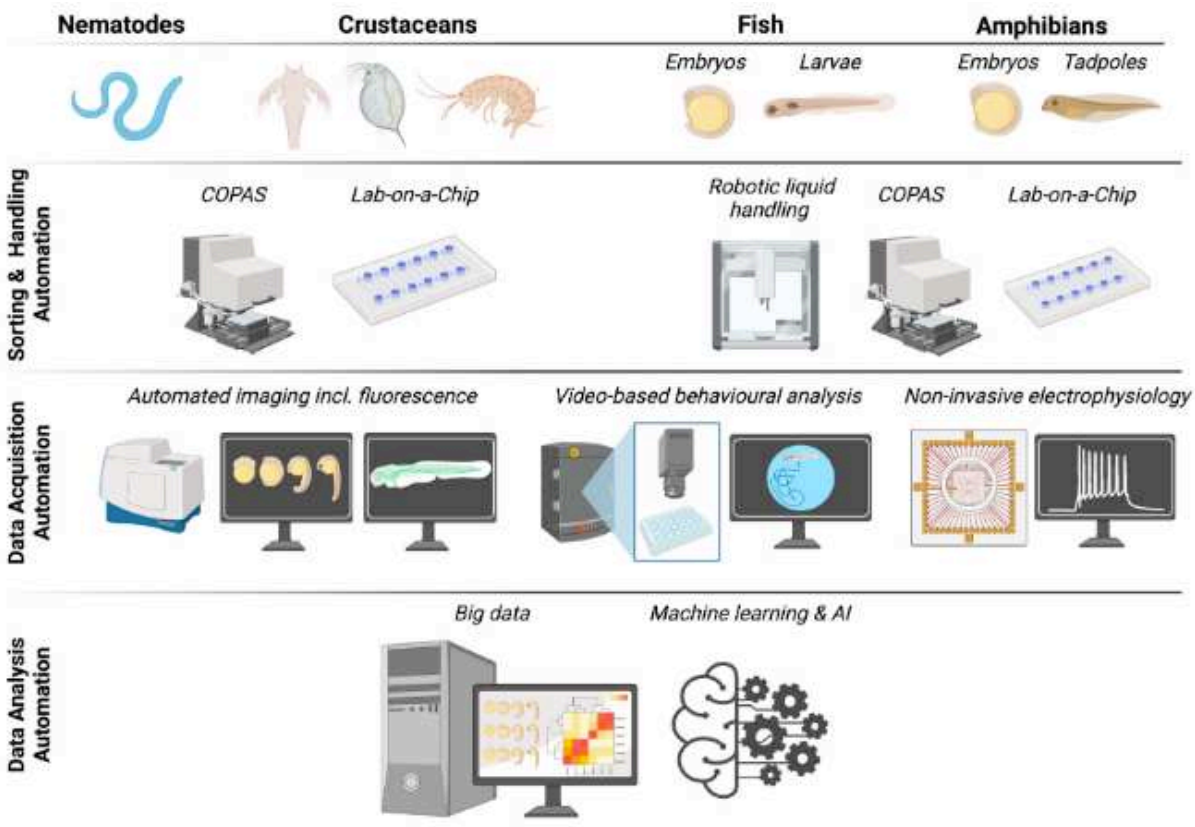


Figure 10: Exemples de méthodologies appliquées en écotoxicologie et qui utilisent des espèces aquatiques. La plupart des techniques sont adaptées à des petits organismes (nématodes, daphnies, gammares), et aux stades embryonnaires. Les techniques incluent de l'imagerie, de l'analyse comportementale et de l'électrophysiologie. Le développement d'algorithmes de traitements automatisés des données est encore peu développé. D'après Wlodkowic et Jansen (2022).

Encore plus loin grâce à la cognition ?

Un des derniers éléments clés qui permet d'évaluer la neurotoxicité des polluants est **l'étude de la cognition**. Je discute de la définition du terme cognition au paragraphe suivant (1.5). S'intéresser à la cognition revient à étudier la manière dont un organisme va manipuler des informations, que ce soit pendant une **phase d'acquisition, de stockage ou d'utilisation de cette information**. Les capacités cognitives sont les aspects les moins étudiés en écotoxicologie, alors que les comportements relatifs à la cognition représentent les fonctions de plus haut niveau du système nerveux (Truong et al. 2014). Ces capacités sont donc les plus susceptibles d'être affectées par des doses sous-létales de plusieurs types de polluants comme les métaux lourds ou les microplastiques (Bownik et Wlodkowic 2021; Pasquini et al. 2024; Monchanin et al.

2024). Aujourd'hui, de nombreux auteurs tentent de valoriser l'étude des capacités cognitives dans des contextes différents de la biologie expérimentale. Par exemple, plusieurs auteurs suggèrent d'étudier la cognition en lien avec le bien-être animal (MacDonald et Ritvo 2016), les enjeux de conservation (Buchholz 2007; Greggor et al. 2014; Roth et Krochmal 2015), ou les questions d'écologie comportementale (Pritchard et al. 2016; Morand-Ferron et al. 2016).

Pour aller plus loin que l'effet de polluants aux doses létales et répondre aux enjeux de rapidité et d'efficacité actuels, il semble opportun de développer des indicateurs biologiques en utilisant des modèles invertébrés et en étudiant leur cognition via des analyses comportementales. Ces méthodologies, couplées avec les avancées techniques actuelles, permettent d'étudier les altérations neurotoxiques des polluants, d'être précis, quantitatifs, à faible coût, et pertinents vis-à-vis du contexte écologique. **Dans ce travail, j'ai profité de ces développements récents et du développement d'un nouvel indicateur biologique pour étudier la cognition d'un invertébré aquatique.**

La cognition - les bases de l'apprentissage et de la mémorisation

Définition(s) des notions clés à l'étude des capacités cognitives

Avant de pouvoir définir les problématiques et enjeux de ce travail de thèse, il est primordial de bien définir les **notions de comportement, de cognition, d'apprentissage et mémorisation**. D'une part, il est nécessaire de s'accorder sur une tentative cohérente de représenter la réalité. Il est également nécessaire d'être précis dans les termes à employer, en particulier en biologie expérimentale. D'autre part, chacune de ces notions sont sujettes à débat (Keijzer 2021).

La notion de **comportement** semble la plus évidente. **C'est ce qu'un animal « fait ».** Pourtant, il y a une grande différence entre cette définition simpliste et une définition plus spécifique, comme **« une réponse à des stimuli internes et externes,**

suite à l'intégration de composantes sensorielles, neurales, endocriniennes et effectrices ». Alors, qu'est-ce que c'est réellement, le comportement ? Levitis et al. (2009) ont répondu à cette question en analysant une série de définitions du comportement, puis en interviewant 174 membres de sociétés savantes, plus ou moins spécialistes du comportement. Leurs résultats surprenants font part de nombreux désaccords et plus de la moitié des chercheurs qui font preuve de contradictions. Ainsi, les auteurs concluent en proposant une définition qui respecte quatre critères : **opérationnelle**, elle indique des caractéristiques précises de ce qui répond ou non à cette définition ; **essentielle**, sa signification est compréhensible en elle-même ; **largement applicable** : elle s'applique dans différents domaines scientifiques ; **succincte**, elle est dépourvue d'éléments descriptifs, explicatifs, de clauses ou de réserves non essentiels à la compréhension. La définition qu'ils proposent est donc : **le comportement est l'ensemble des réponses coordonnées (actions ou inactions) d'organismes vivants entiers (à l'échelle d'individus ou de groupes) à un stimulus interne et/ou externe, en excluant les réponses que l'on peut plus facilement interpréter comme des changements développementaux.**

Plus récemment, Calhoun et El Hady (2023) ont également tenté de répondre à cette question et ont analysé 25 définitions différentes du comportement. Ils ont proposé à des chercheurs de plusieurs disciplines des questions qui entrainent des réflexions stimulantes telles que : peut-on parler de comportement pour : un programme informatique ? Une éponge qui filtre l'eau ? Un bébé qui urine ? Une impulsion neuronale ? En résumé, chaque discipline académique emploie toujours sa propre définition, qui varie sur un spectre de six définitions. Ainsi, nous choisirons dans ce travail la définition proposée par Levitis et al. (2009).

La **cognition** regroupe également un ensemble complexe à résumer, comme en témoigne l'article paru en 2019 dans le journal *Current Biology* qui s'intitule « *What is cognition ?* ». Cette publication recueille l'opinion de onze chercheurs sur leur vision de la cognition, et chaque réponse est bien sûr différente (Bayne et al. 2019). Néanmoins, une complémentarité se retrouve autour d'un concept, qui comprend des **activités et des processus et qui concernent l'acquisition, le stockage, la récupération et le traitement de l'information.** Cette définition de la cognition est très large afin

d'englober une variété de supports et de mécanismes et est compatible avec les recherches menées dans de nombreux domaines, notamment la psychologie, l'éthologie, l'écologie sensorielle, la génétique ou les neurosciences. Malgré une composante opérationnelle qui pourrait être précisée, cette définition permet néanmoins d'être essentielle, largement applicable et succincte. Elle traduit l'essor fulgurant de ce concept et l'étendue des travaux qui y réfèrent. D'expériences de laboratoires, comme déjà en 1913 où C.H. Turner discutait de la capacité d'une blatte à faire preuve de volonté, à des approches de terrain, les sciences cognitives explorent et comparent l'esprit des animaux (Turner 1913; Heyes 2012; Pritchard et al. 2016). De même qu'en 1974, T. Nagel se demandait quel effet cela fait d'être une chauve-souris, les avancées dans ce vaste ensemble permettent à Ros-Rocher et Brunet (2023) de se demander aujourd'hui quel effet cela fait d'être un choanoflagellé.

Définir la notion **d'apprentissage** est un exercice qui suscite de nombreuses réactions. D'une part, c'est un exercice frustrant. En effet, en tant que différence entre un instant t et un instant t+1, on ne peut pas observer directement l'apprentissage en tant que processus. En particulier, en observant le comportement, on peut simplement observer une **modification comportementale entre un moment initial et un moment final**. Cela implique que les recherches sur l'apprentissage doivent comporter une série d'expériences soigneusement contrôlées afin d'exclure toute alternative à l'apprentissage (Dukas 2008). Ainsi, de nombreux chercheurs ont mené à bien des expériences afin de définir des organismes qui apprennent, tout en tentant de définir ce qu'implique le fait d'apprendre. La définition employée dans ce travail de thèse provient d'un exemple historique. En 1943, W. H. Thorpe définit **l'apprentissage comme un processus, au sein d'un organisme, qui produit un changement adaptatif dans le comportement individuel, en résultat à une expérience** (Thorpe 1943). Cette définition semble essentielle, largement applicable et succincte, mais manque certainement de composante opérationnelle, car elle n'explicite pas par exemple si la modification du comportement est nécessaire *de facto*. Est-ce que le changement d'un processus interne seulement correspond à un apprentissage ? Cependant, elle précise déjà que ce processus est à l'échelle individuelle. De plus, elle inclut un changement, donc une comparaison entre un état antérieur, ancien, et postérieur, nouveau. Elle inclut

également la notion d'expérience, qui introduit la présence d'informations acquises et donc l'existence d'une mémoire comme lieu de stockage de cette information. Enfin, elle inclut le principe adaptatif qui a fait l'objet de nombreuses publications, comme en témoigne la revue de Burgos (2018). Dans nos travaux, il est important de considérer la valeur adaptative de l'apprentissage afin de mettre en évidence ce phénomène par rapport à d'autres, car la présence de comportements non-adaptatifs pourraient être des témoins de la présence de polluants (Jacquin et al. 2020). Par exemple, la fatigue motrice est une modification du comportement résultat de contraintes physiologiques, et n'est donc pas comprise dans la définition choisie.

D'autre part, le vif débat qui anime les différentes communautés scientifiques depuis deux siècles en font un domaine extrêmement riche, vivant et passionnant (Barron et al. 2015). Qu'est-ce que l'apprentissage ? Quels sont les constituants biologiques élémentaires qui permettent l'apprentissage ? Quels sont les organismes capables d'apprendre et comment font-ils ? Les débats autour de ces questions et d'autres sont si intenses que certains auteurs parlent d'une véritable guerre de la cognition. Comme dit F. Adams, c'est heureusement une guerre entre universitaires, il est donc probable que personne en soit ne sera blessé, mais c'est tout de même une guerre ! (Adams 2018). Les enjeux sont d'abord autour de l'explication théorique du phénomène d'association entre deux évènements. Les théories reposent notamment sur les processus d'attention, sur la mémoire ou sur la comparaison entre apprentissage et performance. Les enjeux de ces débats sont aussi sur la méthodologie employée, en argumentant sur l'importance de plusieurs phénomènes comme le conditionnement de 1er ordre, 2ème ordre, le phénomène de masquage, etc (Loy et al. 2021). Enfin, les questions liées à l'apprentissage émergent directement des modèles employés et de leurs caractéristiques neurobiologiques, notamment chez les protistes (Gershman et al. 2021), les échinodermes (Freas et Cheng 2022), les myxomycètes (Dussutour 2021), les cnidaires (Botton-Amiot et al. 2023) ou les plantes (Gagliano et al. 2018). Afin de voir comment étudier ces concepts, il est essentiel de décrire maintenant les différents types d'apprentissages.

Apprentissage associatif

Comme son nom l'indique, ce type d'apprentissage crée un lien, une association, entre deux évènements ou plus. Deux grandes catégories d'apprentissage associatif existent.

D'abord, le **conditionnement classique** (pavlovien) permet d'associer un stimulus à priori neutre à une réponse conditionnelle. Par exemple, une récente étude s'est intéressée à l'origine évolutive de l'apprentissage associatif en travaillant avec un modèle de cnidaire, l'anémone étoilée *Nematostella vectensis,* qui ne possède pas de système nerveux central (Botton-Amiot et al. 2023). Dans leur étude, les auteurs ont utilisé un choc électrique (stimulus aversif ou inconditionnel) qui déclenche une forte rétraction de la région orale et des tentacules de l'anémone (réponse inconditionnelle), qu'ils ont associé à une source lumineuse qui n'avait pas d'effet à priori (stimulus neutre ou conditionnel). Après un entrainement de 20 répétitions des deux stimuli ensemble, un test montrait qu'une majorité d'individus se rétractaient à l'apparition de la lumière seule (réponse conditionnelle), significativement plus que des groupes contrôles qui ne présentaient pas cette association des deux stimuli.

Dans le **conditionnement opérant**, c'est directement le comportement de l'individu entrainé qui va faire office de stimulus. Celui-ci peut alors être renforcé ou puni. Par exemple, les larves de fourmillions sont connues pour créer des pièges en forme de cône renversé dans le sable. Lorsqu'une proie, comme une fourmi, tombe dans le piège, elle aura du mal à s'en échapper, laissant du temps au fourmilion caché dans le sable d'en faire son repas. En 2021, Miler et Scharf ont établi une première preuve de conditionnement opérant chez les fourmillions. Dans cette étude, la punition était une chute de 10 cm sur une table, et le renforcement positif était la possibilité de s'enterrer dans le sable. Dans un labyrinthe en Y, les auteurs ont successivement renforcé le comportement de s'enterrer dans le sable dans la direction opposée à celle que le fourmilion avait initialement choisi. Si le fourmilion allait dans la mauvaise direction, il tombait sur la table, alors que s'il choisissait la bonne direction, il pouvait s'enterrer. Ainsi, les individus ont progressivement tourné de plus en plus dans la direction opposée à leur préférence initiale afin d'accéder de plus en plus souvent au sable pour s'enterrer Miler et Scharf (2021).

D'autres paradigmes existent et permettent d'explorer d'autres capacités d'apprentissage chez les animaux. Par exemple, les apprentissages où la valence positive ou négative des stimuli est inversée (c'est-à-dire, les apprentissages non élémentaires), permettent d'explorer des capacités cognitives très complexes, en particulier chez les invertébrés. Ces concepts appréhendent des apprentissages de numérosité, de similarité, spatiaux, de contexte, ou configuraux (Giurfa 2003, 2015; Avarguès-Weber et al. 2012). Chez les insectes, ces paradigmes expérimentaux ont été majoritairement développés chez l'abeille et la drosophile, via leur longue histoire comme modèles expérimentaux et la connaissance détaillée de leur biologie (Meller et Davis 1996). Cependant, d'autres modèles invertébrés sont également étudiés de manière approfondie comme les nématodes (Amano et Maruyama 2011), les gastéropodes (Hawkins et Byrne 2015), les céphalopodes (Dickel et al. 2000), les crabes (Tomsic et Romano 2013), les fourmis (Piqueret et al. 2019), les parasitoïdes (Kishani Farahani et al. 2021) ou les crickets (Mizunami et al. 2013).

Apprentissage non-associatif

Les apprentissages non-associatifs correspondent à une diminution (**habituation**) ou à une augmentation (**sensibilisation**) d'une réponse comportementale à la présence répétée ou prolongée d'un stimulus. Ils sont basés sur le constat qu'un animal est en permanence confronté à des stimuli différents, et qu'il doit être attentif à ceux qui sont importants, alors qu'il doit ignorer ceux qui ne le sont pas. L'habituation est en théorie ce qu'il se passe pour tous les comportements, pour tous les organismes (Thompson 2009). Pour illustrer ce phénomène avec un exemple historique, voici une fable d'Esope :

« Un renard n'avait jamais vu de lion. Or le hasard le mit un jour en face de ce fauve. Comme il le voyait pour la première fois, il eut une telle frayeur qu'il faillit en mourir. L'ayant rencontré une deuxième fois, il eut peur encore, mais pas autant que la première fois. Mais à la troisième fois qu'il le vit, il s'enhardit jusqu'à s'en approcher et à causer avec lui ». Fable d'Ésope, VI^{ème} *siècle av. J. -C.*

L'étude de l'habituation chez divers organismes, des amibes aux humains, a connu un essor à partir des années 1900. D'autres termes étaient alors employés comme « acclimatation, accommodation, adaptation négative, fatigue, extinction ou inactivation stimulatrice » (Thompson 2009). Dans une revue en 1943, Harris souligne l'existence de ce phénomène et propose l'emploi du terme habituation (Harris 1943). En 1956, une publication va être très influente et faire grandir ce domaine d'étude. En mesurant l'activité cérébrale de chats via des implants sous-corticaux durant leur sommeil naturel, les auteurs ont observé la réponse des chats face à un stimulus acoustique (bruit émis par un haut-parleur à une fréquence précise). Trois éléments majeurs ont été révélés. Avec la répétition du stimulus, le réveil des chats devenait de plus en plus court jusqu'à disparaître ; la réponse d'éveil se rétablissait spontanément après plusieurs heures ; et des bruits d'une autre fréquence provoquaient une forte réaction des chats (Sharpless et Jasper 1956). En 1966, Thompson et Spencer établissent une revue des études qui portent sur l'habituation et proposent **9 critères** afin de l'évaluer. Ces critères sont revus par Rankin et al. (2009), qui en ajoute un dixième.

Le 1er critère décrit simplement l'habituation et les suivants permettent de distinguer l'habituation d'autres phénomènes non spécifiques à l'apprentissage. Notamment, les critères 2 et 8 semblent les plus importants afin de prouver que l'on est bien en présence d'habituation. La **réponse spontanée** (2ème critère) exprime une augmentation de la réponse après l'entrainement (la phase d'apparition répétée du stimulus) et une phase de repos. Si on propose alors un stimulus d'une nouvelle modalité, puis à nouveau le stimulus initial, la **déshabituation** (8ème critère) indique que la réponse au stimulus initial sera augmentée. Dans le même sens, la réponse au nouveau stimulus sera augmentée en fonction de son degré de nouveauté (**spécificité**, 7ème critère). Si le stimulus est représenté dans une modalité sensorielle différente, la réponse est augmentée, sinon, la réponse est comparable à celle de la fin de l'entrainement. La répétition de ce nouveau stimulus peut également provoquer de l'habituation (9ème critère). Par ailleurs, les critères 3, 4, 5 et 6 sont liés aux paramètres qui influencent l'habituation. L'augmentation en fréquence (4ème critère) et en intensité

(5ème critère) du stimulus modulent la rapidité d'un organisme à s'habituer. Si on fait plusieurs séries d'entrainements, l'habituation sera plus rapide (3ème critère) et si on augmente le nombre d'essais lors d'un entrainement, on augmente le délai avant la réponse spontanée (6ème critère). Enfin, l'habituation à **long terme** indique que la réponse à long terme peut être différente : ré-habituation plus rapide, réponse initiale plus faible, réponse moyenne plus faible, réponses moins fréquentes. Ce dernier critère est également essentiel car il sous-entend que l'habituation à court terme et à long terme impliquent des mécanismes cellulaires qualitativement différents.

Modèles et mécanismes sous-jacents

Afin d'expliquer les mécanismes qui sous-tendent le phénomène d'habituation, de nombreuses études ont été menées et de nombreuses théories ont vu le jour (Thompson 2009). Parmi celles-ci figure la théorie « *Dual process* » de Groves et Thompson, qui implique, comme son nom l'indique, deux processus en parallèle et indépendants, qui s'affrontent pour déterminer le résultat comportemental final après une série de stimuli. Le premier, nommé « voie stimulus-réponse », est la voie la plus directe à travers le système nerveux central entre le stimulus et la réponse, alors que la seconde, « l'état », détermine le niveau de réactivité de l'organisme (Figure 11). D'après les auteurs, il existe trois types d'interneurones dans ce modèle, dont un type qui est non-plastique, c'est-à-dire qu'il ne montre pas de changement de réponse. En revanche, deux types d'interneurones sont plastiques : le type H qui montre une diminution de son activité et le type S qui montre une augmentation initiale avant une diminution de son activité. Lorsqu'un stimulus est présenté avec répétition, la diminution de réponse est due à la diminution de l'activité de neurones H qui module le niveau de réponse. Si le stimulus dépasse le seuil de sensibilité des neurones S, cela entraine une augmentation de la réponse. Cette théorie propose donc que la déshabituation n'est pas une rupture de l'habituation mais plutôt un processus distinct, en parallèle de la voie Stimulus-réponse (Prescott 1998).

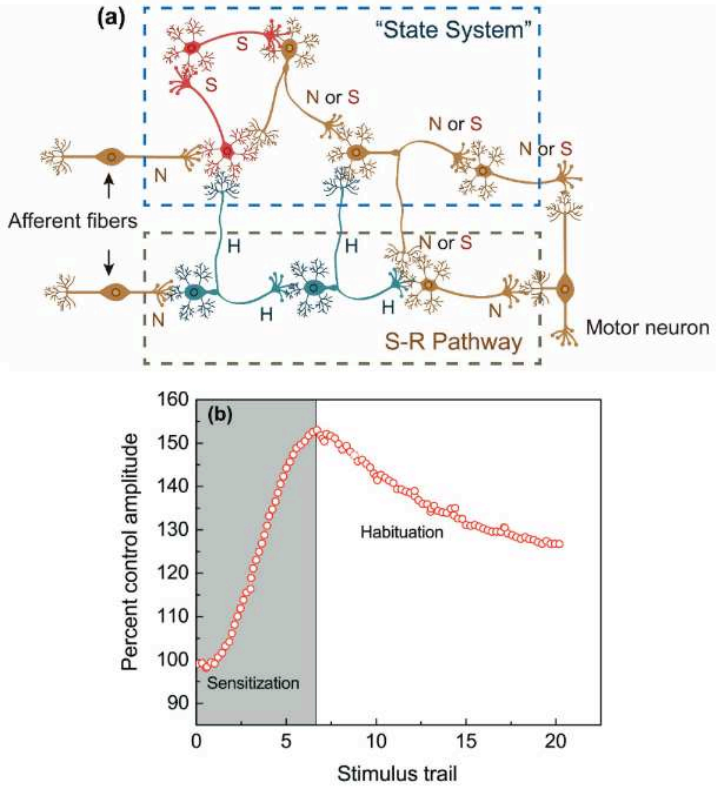


Figure 11: (a) Schéma illustrant la théorie « Dual process » de Groves et Thompson. N = synapses non-plastiques. H = synapses qui s'habituent. S = synapses qui vont se sensibiliser. L'information liée au stimulus se transmet par des fibres afférentes, selon les deux voies qui interagissent, jusqu'aux motoneurones qui vont produire le comportement. (b) Courbe de réponse typique à la répétition d'un stimulus, dans le cadre de l'expérience du réflexe de flexion du membre postérieur chez le chat de Grooves et Thompson. Après une phase de sensibilisation, la réponse diminue. D'après Shi et al. (2017).

L'habituation est un apprentissage qui est toujours très étudié, comme en témoigne les 50 000 publications concernées par ce sujet entre 2000 et 2005 (Thompson 2009). Bien qu'ayant plus de 50 ans, la théorie de Groves et Thompson et d'autres sont encore débattues, car les mécanismes se révèlent souvent plus complexes (Schmid et al. 2015; Lamiré et al. 2023). De plus, les débats portent sur l'habituation en tant que processus à l'échelle cellulaire, comme chez les cellules PC12. Cette lignée de cellules de phéochromocytome (tumeur de la médullosurrénale chez le rat) a été étudiée dans le cadre de sa réponse à une stimulation d'ions potassium (K+) ou d'acétylcholine (ACh). Sans parler de comportement ici, ces cellules sécrètent de la noradrénaline, et diminuent leur sécrétion à la stimulation répétée de K+ ou d'ACh. De plus, ces cellules rétablissent leur taux de sécrétion après une période de repos et présentent une spécificité, un effet fréquence et intensité du stimulus, une habituation à court et long terme ainsi qu'une déshabituation. En bref, elles respectent tous les critères établis pour

valider l'habituation et sont donc des modèles très prometteurs pour étudier les bases physico-chimiques de l'habituation (Thompson 2009; Dussutour 2021).

Les différents types de mémoires

Pour terminer sur les notions à aborder dans ce travail, la possibilité d'apprentissage assume implicitement l'existence d'une **mémoire**, c'est-à-dire d'une **capacité à retenir les nouvelles informations acquises pendant une période**, qui peut être très courte (mémoire à court terme) ou plus longue (mémoire à long terme). Ces deux grands types de mémoire possèdent des mécanismes différents, découverts depuis au moins 25 ans. Par exemple, Izquierdo et al. (1998) ont montré que les processus pour former la mémoire à court terme pouvaient être bloqués tout en gardant intacte une mémoire à long terme chez le rat (Izquierdo et al. 1998). Au contraire des millions de neurones trouvés chez les mammifères, les invertébrés possèdent des petits cerveaux, composés de quelques centaines ou milliers de neurones. Ils sont donc appropriés pour étudier les constituants principaux de la mémoire, et ont permis de faire des découvertes significatives, notamment sur les différents types de mémoire, leurs mécanismes et les circuits impliqués (Menzel et Benjamin 2013).

Avec le crabe, l'aplysie et l'abeille, la drosophile fait partie des modèles où l'on connait le mieux le fonctionnement de la mémoire, grâce au développement de mutants possédant des transgènes permettant d'activer ou d'éteindre des sets spécifiques de neurones pendant ou après un apprentissage, ainsi que des techniques d'imagerie cellulaire fonctionnelles permettant d'observer l'activité de sets de neurones grâce à l'activation d'une protéine fluorescente (GFP) (Tomchik et Davis 2013). La drosophile présente plusieurs types de mémoires, d'engrammes, qui se distinguent par les mécanismes sous-jacents l'expression de ces mémoires. Afin de déterminer les propriétés de ces mémoires, il faut 1) observer une réponse comportementale précise après un apprentissage et 2) trouver un support physiologique c'est-à-dire une trace mnésique (Davis 2011). Pour la première étape, il est nécessaire de mettre en place un paradigme expérimental comprenant une ou plusieurs apparitions d'un stimulus, et mesurer le plus précisément possible la réponse comportementale des individus soumis à ce stimulus. Afin de mettre en évidence des potentiels candidats pour

expliquer ces différents types de mémoire, les chercheurs utilisent des **anesthésiants ou du cycloheximide**, un antifongique qui bloque la biosynthèse de protéines chez les eucaryotes (Mileusnic 2004). Pour la seconde étape, les chercheurs utilisent des techniques d'imagerie in vivo, et ciblent les zones neuronales susceptibles d'avoir été modifiées pendant l'apprentissage, et qui seraient donc la représentation physiologique de la mémoire dans le cerveau. Chez la drosophile, l'apprentissage le plus utilisé est le conditionnement olfactif, et permet l'apparition de plusieurs mémoires localisées dans les lobes antennaires (AL) et les corps pédonculés (MB) (Busto et al. 2010).

Après conditionnement, la drosophile exprime immédiatement une mémoire à court terme (« *Short-term memory* », STM), qui décline rapidement. Cette STM se retrouve dans les neurones de projection des lobes antennaires (PN), qui reçoivent les informations des neurones olfactifs des antennes et projettent vers les corps pédonculés (MB) et la corne antérieure (LH, Figure 12). La STM est également révélée dans les neurones latéraux pairs antérieurs (APL) et les neurones dopaminergiques (DA) qui projettent également vers les MB, ainsi que dans les neurones α'/β' des MB. La mémoire intermédiaire (« *Intermediate-term memory* », ITM) se trouve sous forme de trace entre 30 et 60 min après entrainement, dans les neurones dorsaux postérieurs médians (DPM). Elle requiert la synthèse de protéines existantes mais pas d'ARN. Deux types de mémoire à long terme (« *Long-term memory* », LTM), dont une en phase tardive (« *Late-phase long-term memory* », LP-LTM), ont la particularité de dépendre de la synthèse de nouvelles protéines et d'ARN (Chen et al. 2012). La LTM se retrouve principalement dans les neurones α/β des MB, et la LP-LTM se retrouve dans les neurones γ entre 18 et 48h après conditionnement. On retrouve enfin des traces de synthèse de protéine pour la LTM dans les lobes antennaires (Figure 12, Tomchik et Davis 2013).

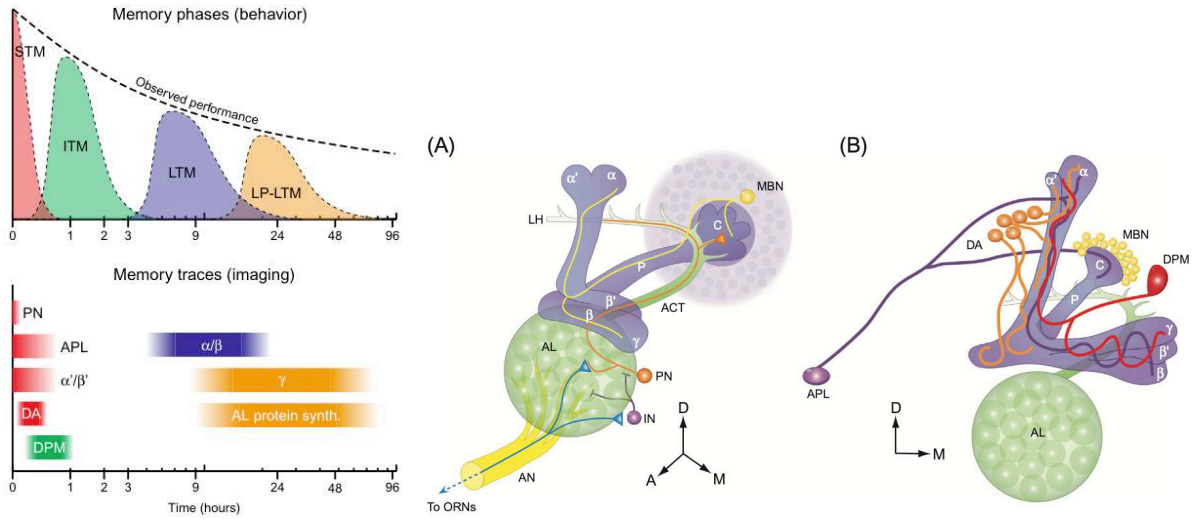


Figure 12: A) Différents types de mémoire et les traces mnésiques associées. STM : mémoire à court terme. ITM : mémoire intermédiaire. LTM : mémoire à long terme. LP : phase tardive. PN : neurones de projection des lobes. APL : neurones latéraux pairs antérieurs. α'/β' : neurones α'/β'. DA : neurones dopaminergiques. DPM : neurones dorsaux postérieurs médians. α/β : neurones α/β. γ : neurones γ. ACT : tractus antenno-cerebral. AL protein synth. : synthèse de protéine dans les lobes antennaires. B) Structure de la voie olfactive chez la drosophile. ORN : neurones récepteurs olfactifs. AN : nerf antennaire. AL : lobe antennaire. IN : interneurones. PN : neurones de projection. MBN : neurones des corps pédonculés. LH : corne latérale. C : Calice. P : pédoncule. APL : neurone latéral antérieur. DPM : neurone dorsal postérieur médian. Abréviations directionnelles : A, antérieur, D, dorsal, M, médial. D'après Tomchik et Davis (2013).

La formation de la mémoire est également dépendante de **l'intervalle entre les essais** durant l'apprentissage. En effet, les chercheurs établissent empiriquement deux types d'entrainements : **massés**, lorsque les intervalles sont très courts, et **espacés**, lorsque les intervalles sont plus longs (à partir de plusieurs dizaines de secondes). Chez la drosophile, le conditionnement espacé produit les mémoires décrites ci-dessus, alors que le conditionnement massé produit une mémoire indépendante de la synthèse de protéines (« *Protein-synthesis independant* », PSI) et résistante à l'anesthésie (« *Anesthesia Resistant Memory* », ARM), dont certaines caractéristiques sont encore débattues (Isabel et al. 2004).

Tenter de mieux comprendre les différents types de stockage, rétention, récupération et expression de la mémoire chez les invertébrés est essentiel afin de comprendre les mécanismes évolutifs et pouvoir faire des comparaisons entre espèces. Il est également crucial de comprendre les différents coûts associés aux différentes mémoires, afin de comprendre comment les invertébrés aux vies de courte durée vont

adapter ou non leur comportement face à l'environnement (Lihoreau et al. 2019). De multiples **compromis (*trade-offs*)** se présentent chez ces organismes afin d'investir dans des mémoires couteuses ou non (Thornton et Boogert 2019). Enfin, les invertébrés présentent une incroyable flexibilité comportementale, au sens défini dans les neurosciences comportementales, c'est-à-dire la capacité d'un individu à répondre et ajuster son comportement aux stimuli environnementaux (Coppens et al. 2010).

La cognition est très étudiée chez les invertébrés qui font preuve de capacités d'apprentissage et de mémorisation remarquables. Grâce à des avancées scientifiques et techniques, nous pouvons aujourd'hui mieux comprendre les mécanismes et supports physiologiques de ces capacités, comme en témoigne les travaux sur la drosophile. Parmi les paradigmes expérimentaux, l'habituation est le plus accessible et fournit un support robuste pour poser des questions fondamentales liées aux capacités des organismes à adapter leur comportement vis-à-vis de leur environnement. **Cette thèse n'explore pas les mécanismes qui sous-tendent l'expression des capacités cognitives. Ainsi, le travail présenté ici s'articule principalement autour des phases de perception, d'apprentissage et de mémorisation via l'étude du comportement animal. En particulier, j'ai utilisé l'habituation pour caractériser les performances cognitives d'un modèle invertébré qui, comme la drosophile, appartient à l'ordre des diptères : le moustique, à l'état larvaire**. Comme le renard d'Ésope, les larves de moustique diminuent leur peur face à un stimulus aversif et un des enjeux de cette thèse est de mesurer l'habituation chez ces individus.

La larve de moustique : un modèle prometteur

Biologie des moustiques

Les **moustiques** (famille des Culicidae), « petites mouches » holométaboles, appartiennent à l'ordre des diptères, comme les Drosophilidae dont ils ont divergé il y a environ 250 millions d'années (Mysore et al. 2011). Néanmoins, il a été suggéré qu'environ 56% de leurs gènes sont orthologues. A ce jour, environ 3600 espèces ont été

décrites au niveau mondial, dont seulement quelques centaines (environ 6%) ont besoin de sang (Fang 2010). Certaines espèces sont intéressées par les humains, alors que d'autres préfèrent les amphibiens ou les fourmis. Les moustiques ont colonisé tous les écosystèmes terrestres sur tous les continents (sauf l'Antarctique). Bien qu'un récent article publié dans *Current Biology* présente un fossile d'un moustique mâle qui possède les pièces buccales apparemment adaptées pour se nourrir de sang, ce sont uniquement les femelles qui peuvent se nourrir de sang humain (Azar et al. 2023). En effet, elles ont besoin de protéines contenues dans le sang pour produire leurs œufs, et la majorité de leur régime alimentaire et de celui des mâles provient du nectar et de la sève des plantes. La durée de vie des adultes est comprise entre quelques jours et plusieurs mois, et la phase imaginale est aérienne tandis que les phases larvaires sont aquatiques (Figure 13).

Certaines espèces propagent des agents pathogènes qui ont des conséquences graves et étendues, principalement chez les mammifères. Le moustique est l'espèce qui tue le plus d'humains (725 000 humains par an), quinze fois plus que les serpents, cinquante mille fois plus que les requins. D'autres pathogènes sont transmis par le moustique et peuvent infecter oiseaux, bovins, chiens et chats (Becker et al. 2020). Seules neuf espèces de moustiques sont considérées comme invasives, mais représentent de fortes capacités vectorielles, et s'appuient notamment sur deux stratégies efficaces : la capacité à se reproduire dans des environnements anthropisés et la capacité à produire des œufs résistants à la dessiccation (Juliano et Philip Lounibos 2005).

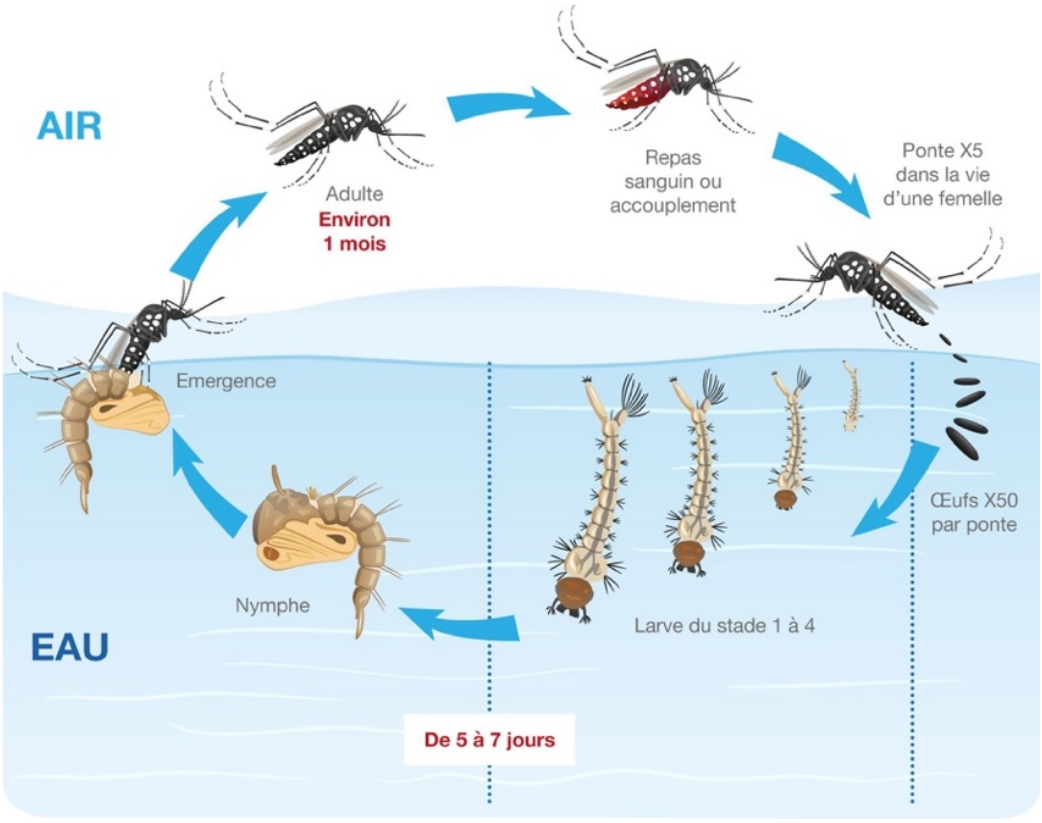


Figure 13: Cycle de vie du moustique. Les femelles pondent leurs œufs directement dans l'eau (*Anopheles*, *Culex*) ou sur un substrat humide (*Aedes*). Les larves vont se développer durant quatre stades larvaires avant de se transformer en pupe (nymphe). Après émergence, les mâles et les femelles s'accouplent, puis les femelles partent à la recherche d'un hôte. D'après : « Le moustique de A à Z, la Réunion ».

Chez le moustique, trois genres sont les plus représentés : le genre ***Culex***, le genre ***Anopheles*** et le genre ***Aedes***. *Culex* et *Aedes* auraient divergé du genre *Anopheles* il y a 120 000 d'années (Figure 14), mais ils présentent un nombre équivalent de neurones (220 000 neurones contre 200 000 chez la drosophile, Raji et Potter 2021). Ces trois genres comprennent les espèces qui ont la plus grande importance médicale en tant que vecteur de maladie. Alors que les espèces du genre *Aedes* sont adaptées à des zones urbaines et périurbaines, celles du genre *Anopheles* sont associés à des habitats aquatiques de plus grande taille, et les deux cas sont présents chez *Culex*. Leur adaptabilité à différents environnements, leur capacité à exploiter des sites d'oviposition variés et leur répartition géographique en font également des modèles biologiques importants (Becker et al. 2020).

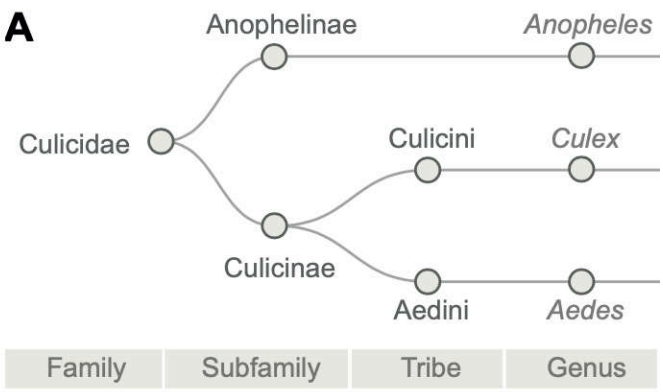


Figure 14: Phylogénie simplifiée des trois genres *Anopheles, Culex* et *Aedes*. D'après Lutz et al. (2020).

Dans leur environnement, les moustiques sont capables d'exploiter plusieurs types d'informations et les intégrer de façon multimodale afin de s'orienter dans l'espace, notamment en utilisant des informations visuelles, olfactives, thermiques et mécaniques (Lazzari 2020). Ainsi, ils sont l'objet d'un grand nombre d'études dans des domaines variés, du séquençage de leur génome jusqu'à la modélisation des dynamiques de population (Holt et al. 2002; Cummins et al. 2012). En particulier, la lutte contre les maladies transmises par les adultes, leur caractère invasif en lien avec le changement climatique, leur rôle en tant que maillon de la chaine trophique mais également leur potentielle résistance face aux insecticides sont autant d'enjeux qui nécessitent des efforts de recherche (Carrasco 2019). Pourtant, la majorité des études qui s'intéressent à l'état larvaire portent sur l'effet de pesticides sur leur survie (Ganesan et al. 2023). En particulier, les recherches sont rares concernant l'étude de la cognition des larves de moustique (Bui et al. 2019; Lutz et al. 2020).

Écologie des larves de moustique

Les larves de moustique sont composées de trois parties : tête, portant les yeux et les pièces buccales, thorax, qui comprend le prothorax, mésothorax et métathorax, pourvus de soies, et l'abdomen, comprenant entre 9 et 10 segments. Les Culicinae (*Culex* et *Aedes*) possèdent un siphon, un tube d'air relié à leur 8ème segment qui flotte à la surface et qui comprend les spiracles, les seuls orifices externes fonctionnels qui servent aux larves à exercer une respiration métapneustique, c'est-à-dire à respirer à la

surface de l'eau. Pourtant, une récente étude montre que la respiration chez les espèces *Aedes aegypti* et *Aedes albopictus* peut se faire également sous l'eau, ayant d'importantes conséquences pour les techniques de contrôle des populations par asphyxie des larves (Alvarez-Costa et al. 2023). Chez les Anophelinae, le siphon est atrophié, extrêmement réduit à la plaque spiraculaire et on dit souvent qu'il est absent (Becker et al. 2020). Le dernier segment abdominal comprend plusieurs types de soies et se termine par des structures flexibles, les papilles anales, qui participent à l'osmorégulation.

La durée de la phase larvaire dépend de plusieurs critères, notamment de l'espèce, de la densité de la population, de la température ou de la quantité de nourriture disponible. Certaines espèces comme *Aedes rusticus* peuvent passer l'hiver sous la glace et rester jusqu'à trois mois en phase larvaire alors qu'il ne faut que quelques jours pour le développement d'*Aedes aegypti* en région tropicale (Becker et al. 2020). Pareillement, les larves de moustique vivent dans un large éventail d'habitats et exploitent différents types de nourritures, en particulier des microorganismes et des détritus végétaux (Afify et Galizia 2015). Les moustiques *Aedes* sont capables de se développer dans de tout petits espaces aquatiques éphémères (empreintes d'animaux, coquilles de mollusques, réservoirs de broméliacées, pots de fleurs) ainsi que des milieux soumis à des polluants, alors que les *Anopheles* requièrent de plus grandes étendues d'eau permanentes telles que des étangs, des marais, des marécages ou des champs agricoles (Clements 1992; Shaalan et Canyon 2009; Chandrasegaran et al. 2020).

Bien que les larves soient toutes contraintes à vivre entre la surface et le fond de l'eau, les genres *Aedes, Culex et Anopheles* diffèrent dans leur comportement. Les éthogrammes qui tentent d'identifier le nombre et la qualité de ces comportements distinguent usuellement quatre types d'activités, chacune associée à une ou plusieurs fonctions. Tout d'abord, les larves présentent une **phase de repos** caractérisée comme une quiescence au sens d'absence d'activité (ex : Kinney et al. 2014). Cette phase est économe en énergie, leur permet de s'auto-toiletter, de s'engager dans des activités de filtration et leur permet de rester immobiles en réponse à des forces de fluctuations de

l'eau (Skiff et Yee 2014). Les larves s'engagent également dans des **activités de fourragement**, principalement via l'activation de leurs pièces buccales. Ce comportement dépend du type de ressource ou de la morphologie des individus (pour une revue, voir Merritt et al. 1992). Le troisième comportement correspond à une **activité de locomotion**, qui prend des dénominations différentes en fonction des auteurs. Ce comportement est caractérisé par une torsion énergique de l'abdomen des individus, par des flexions latérales afin de se mouvoir dans une enceinte aquatique (Figure 15, Skiff et Yee 2014). Enfin, le quatrième comportement mis en évidence est celui de **décrochage de la surface de l'eau**. Lorsque le siphon respiratoire des larves de moustique se ferme, les larves descendent le long de la colonne d'eau. Ce comportement, associé principalement à une fonction anti-prédation, permet aux individus de se mouvoir verticalement dans leur habitat. Par souci de cohérence, nous nommerons les quatre comportements mentionnés : **repos, fourragement, gigotement et plongée.**

Un facteur majeur qui affecte le comportement d'un organisme aquatique est sa **densité** relative à la densité de l'eau. Ici, les larves *Aedes* ont une densité relative inférieure à 1.0 et vont tendance à flotter, alors que les larves *Culex* et *Anopheles* ont une densité relative supérieure à 1.0, et vont donc couler (Clements 1999). De plus, la position latérale *d'Anopheles* à la surface de l'eau peut également modifier la fréquence et l'amplitude de ses comportements en lien avec l'énergie requise pour se décrocher de la surface de l'eau. Enfin, les espèces du genre *Aedes* sont décrites comme très actives, passant la majorité de leur temps en mouvement, alors que les larves *Culex* et *Anopheles* passent plus de temps au repos (Mellanby 1958; Kasap 1978; Tuno et al. 2004; Skiff et Yee 2014).

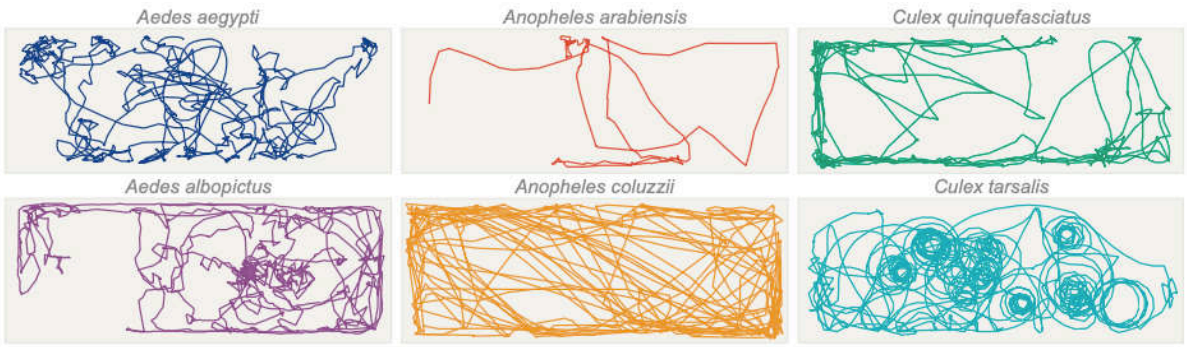


Figure 15: exemple de trajectoires chez les larves de six espèces de moustique appartenant aux trois genres *Aedes, Culex, Anopheles*, dans une eau claire. D'après Lutz et al. (2020).

Cognition des larves de moustique et écologie sensorielle

Dans leur environnement, les larves de moustique sont soumises à plusieurs facteurs qu'elles peuvent percevoir afin d'adapter leur réponse comportementale et améliorer leur fitness. L'un des facteurs les plus importants est la **prédation**, qui se représente par des **vibrations mécaniques** transmises le long de la colonne d'eau ; par des phéromones, substances sémiochimiques ; et par des signaux visuels (contrastes lumineux, ombres). Les larves sont capables de moduler leur comportement en fonction de la présence de prédateurs ou de résidus de congénères, notamment en diminuant leurs gigotements, donc leur distance parcourue et leur vitesse, lorsqu'elles sont exposées à des phéromones de prédateurs (Kesavaraju et Juliano 2004; Ferrari et al. 2007, 2008; Chandrasegaran et al. 2018). Les larves sont également capables de réagir à des stimuli mécaniques, notamment par la plongée, même si des plongées répétées entraine un coût énergétique et une diminution de la survie (Lucas et al. 2001; Tuno et al. 2004). Ces stimuli mécaniques vont être captés par les soies situées sur le corps, la bouche et les antennes (Clements 1999).

Les larves modulent également leur comportement en fonction de la **présence de certains composés chimiques** (pour une revue, voir Afify et Galizia 2015; Lutz et al. 2017). Plusieurs études ont examiné la réponse comportementale des larves de moustique à différentes substances chimiques (Gonzalez et al. 2019). De plus, d'autres études ont exploré la réponse des récepteurs olfactifs situés dans les antennes des

larves de moustique (Xia et al. 2008; Sun et al. 2021). Enfin, une étude de 2019 utilise un modèle de l'espèce *Aedes aegpyti* modifié génétiquement pour exprimer un marqueur calcique, le GCaMP6s, qui assemble entre autres une protéine fluorescente et une protéine dépendante de l'activité du cation Ca^{2+} (Figure 16). Avec des techniques d'imagerie calcique, les auteurs ont pu explorer le rôle de plusieurs composés sur l'activité neuronale chez cette espèce (Bui et al. 2019). De manière générale, les larves semblent attirées par des composés qui représentent les détritus organiques (indole, 2-methylphenol, 4-methylcyclohexanol), et ces composés vont être exprimés dans les récepteurs des neurones olfactifs des antennes. Le comportement des larves est également régulé par leur **état de satiété**, et les trois genres vont montrer différentes stratégies de recherche alimentaire. Par exemple, le genre *Aedes* va avoir tendance à avoir un comportement de recherche non-directif (kinésie), c'est-à-dire qu'il va réduire l'intensité et la fréquence de ses gigotements en présence d'une source d'odeur attirante, et inversement en présence d'une source d'odeur aversive (Lutz et al. 2020).

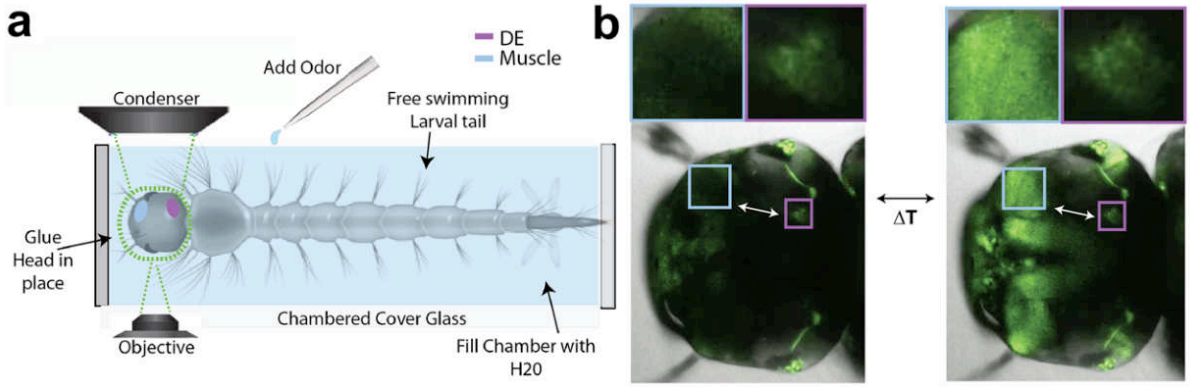


Figure 16: A) Schéma du dispositif permettant de pratiquer de l'imagerie calcique sur les larves de moustique Aedes aegpyti. La tête de la larve est fixée afin de pouvoir positionner l'objectif d'un microscope confocal, alors que le thorax et l'abdomen restent libre de mouvements. L'enceinte est remplie d'eau et après une période de repos, une odeur est injectée. B) L'activité calcique est mesurée dans le deutérocérébrum (DE) ainsi que dans le muscle. Pour comparer l'activité dans ces deux sites, une image est prise pendant le repos puis pendant le pic d'activité après injection de la substance. D'après Bui et al. (2019).

Enfin, les larves réagissent à des **stimuli visuels**, mais peu d'études ont exploré les mécanismes du **système visuel** et les fonctions associées chez les larves de moustique (Mysore et al. 2014; Jenkins et Muskavitch 2015). Dans le système visuel, les larves possèdent deux structures distinctes. D'une part, elles possèdent des **stemmata**

ou yeux simples latéraux qui contiennent des photorécepteurs, cellules sensorielles qui réagissent aux stimuli visuels. Les photorécepteurs contiennent de la rhodopsine ainsi que des opsines, protéines qui réagissent à l'énergie lumineuse. Chez *Aedes aegypti* au stade larvaire, la majorité des photorécepteurs expriment Opsin3 et deux ensembles de petite taille au niveau satellite et centre expriment Opsin7 ((Liu et al. 2022, Figure 17). Les larves possèdent cinq paires de stemmata et expriment deux classes de photorécepteurs (Rocha et al. 2015). Ces stemmata sont présents dès le stade L1, et leurs axones projettent vers le cerveau en développement via le lobe optique. Les spectres d'absorption des stemmata ont été enregistrés entre 300 et 650 nm, avec des pics d'absorbance maximale à 345 nm, 450 nm et 515 nm, proche des spectres d'absorption de l'abeille (Clements 1992).

Au stade L3, les axones des stemmata sont rejoints par les axones du second type de structure, **l'œil adulte composé**, et projettent ensemble vers le lobe optique du cerveau. Au stade nymphal, le lobe optique complexe sera formé avec des neuropiles distincts (Mysore et al. 2014). Les yeux composés, qui commencent donc leur développement à partir du stade L3 larvaire, semblent être fonctionnels durant les derniers stades larvaires et au stade nymphal (Rocha et al. 2015). Les stemmata sont toujours présents chez les adultes, mais glissent petit à petit derrière les yeux composés et sont en état de dégénérescence (Clements 1963).

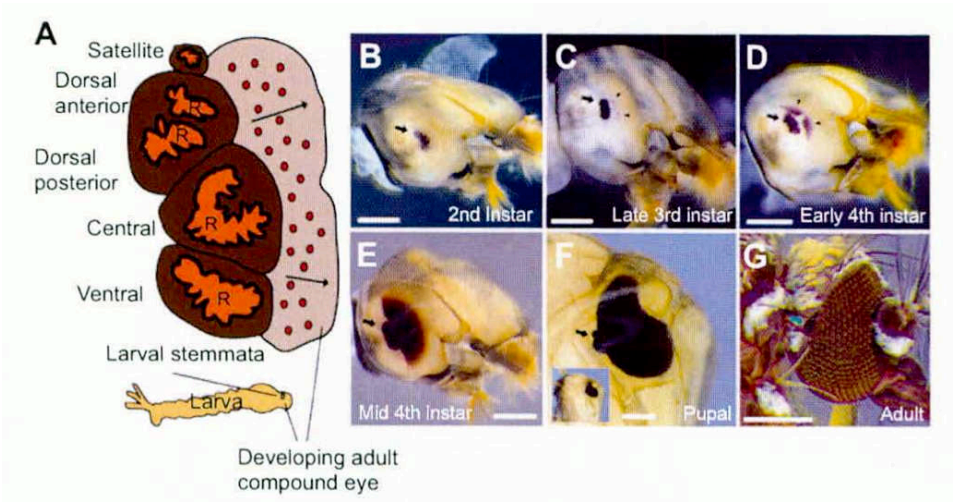


Figure 17: A) Schéma du système visuel *d'Aedes aegypti* durant la phase larvaire. Cinq groupes de photorécepteurs (de satellite à ventral) sont annotés. R : rhabdomères, ensemble des microvillosités d'un photorécepteur. Les flèches noires indiquent le sens de développement de l'œil adulte. B-G) Microphotographies de la tête d'une larve *Aedes aegypti* montrant l'organisation des stemmata et le développement de l'œil composé. Les flèches noires pointent vers les stemmata. B) 2ème stade larvaire.

C) 3ème stade larvaire. D) Début 4ème stade larvaire. E) Milieu 4ème stade larvaire. F) Stade pupe. G) Adulte. D'après Rocha et al. (2015).

D'un point de vue **comportemental**, à chaque stade larvaire, les larves des genres *Aedes, Culex* et *Anopheles* expriment de la **phototaxie négative**, c'est-à-dire qu'elles sont répulsées par les zones lumineuses. (Liu et al. 2022). De plus, bien que le rôle distinct des stemmata et des yeux composés soit mal compris dans la réponse comportementale à un stimulus visuel, au moins une expérience met en évidence qu'ils sont tous les deux capables d'entrainer en réponse de fuite des larves. En 1977, M. Kasap peint les deux structures chez des larves de stade L4 *Aedes aegypti*. La réponse des larves qui n'avait qu'une des deux structures peintes ne différait pas avec le contrôle. Cependant, lorsque les deux structures étaient peintes en même temps, les larves étaient significativement moins réactives au stimulus, indiquant une sensibilité des yeux composés dans la réponse comportementale des larves (Clements 1999; Rocha et al. 2015).

Enfin, les larves de moustique sont **sensibles à différents types de polluants**. Par exemple, des larves *Culex pipiens* exposées à des doses de cuivre à 500 mg/L présentent une vitesse de locomotion diminuée, et cet effet se transmet à la descendance. Le cuivre a également inhibé l'activité enzymatique d'acétylcholinestérase, mais n'a pas modifié la réponse comportementale des larves face à un stimulus aversif simulant un prédateur (Amer et al. 2022). Dans une autre étude, l'herbicide glyphosate, à doses sous-létales, a altéré le comportement de larves de l'espèce *Aedes aegypti* en réponse à un stimulus aversif. En plus d'avoir altéré le comportement, les auteurs ont mis en évidence un effet du glyphosate sur l'apprentissage des larves de moustique (Baglan et al. 2018)

Les larves de moustique possèdent des capacités cognitives développées, qui leur permettent de faire face à des stimuli divers provenant de leur environnement aquatique. En particulier, leur vision leur permet de fuir rapidement un prédateur en se décrochant de la surface de l'eau, en plongeant le long de la colonne d'eau. **Dans cette**

thèse, j'ai observé l'apprentissage des larves de moustique en réponse à un stimulus visuel aversif, simulant un prédateur volant au-dessus de l'eau.

Comment étudier la cognition chez la larve de moustique ?

Avantages des larves de moustique

Les larves de moustique représentent un organisme attrayant afin d'étudier des questions d'écotoxicologie. En effet, leur présence, abondance, et fort enjeu sanitaire partout sur Terre rendent possible des comparaisons à grande échelle. Leur biologie et taxonomie est bien connue, et alors que les adultes dispersent, les larves sont contraintes dans un écosystème donné. Dans leur écosystème aquatique, les larves vont être soumises à divers processus écosystémiques et vont interagir avec d'autres organismes (Rejmánková et al. 2006). Chaque espèce présente des affinités avec des propriétés physico-chimiques de l'eau (Avramov et al. 2023). De plus, les larves sont sensibles aux polluants, mais sont considérées comme relativement résistantes à des conditions dégradées, indiquant potentiellement des effets importants pour les autres organismes vivant dans les mêmes écosystèmes (Baglan et al. 2018; Amer et al. 2022). Les larves de moustique sont associées à des prédateurs, et présentent un panel comportemental largement étudié, comprenant notamment cette réponse comportementale de plongée en réponse à un stimulus aversif. Certaines espèces vont avoir tendance à se nourrir en filtrant les microorganismes de surface alors que d'autres vont se nourrir plutôt dans le substrat. Ainsi, les larves pourraient indiquer la présence de polluants hydrophobes qui ont tendance à être adsorbés dans le susbtrat ou de polluants à forte solubilité qui vont plutôt être présents dans la colonne d'eau (Merritt et al. 1992). Enfin, les moustiques sont des espèces prolifiques, permettant facilement d'élever et de maintenir un grand nombre d'individus. Chez certaines espèces (genre *Aedes* notamment), les œufs sont résistants à la dessiccation jusqu'à plusieurs mois et peuvent être stockés sous une forme déshydratée. Il suffit alors de faire éclore les œufs en même temps pour synchroniser les cycles de développement. Leur courte durée de vie permet également de faire des études multigénérationnelles.

Protocoles d'étude de l'apprentissage chez la larve de moustique

Afin d'explorer les capacités d'apprentissage chez la larve de moustique, les modalités olfactive, visuelle et mécano-sensorielle ont été exploitées, quasi-exclusivement dans le cadre **d'apprentissage par habituation.** Pour illustrer ce phénomène, déjà en 1911, **Holmes** écrivait :

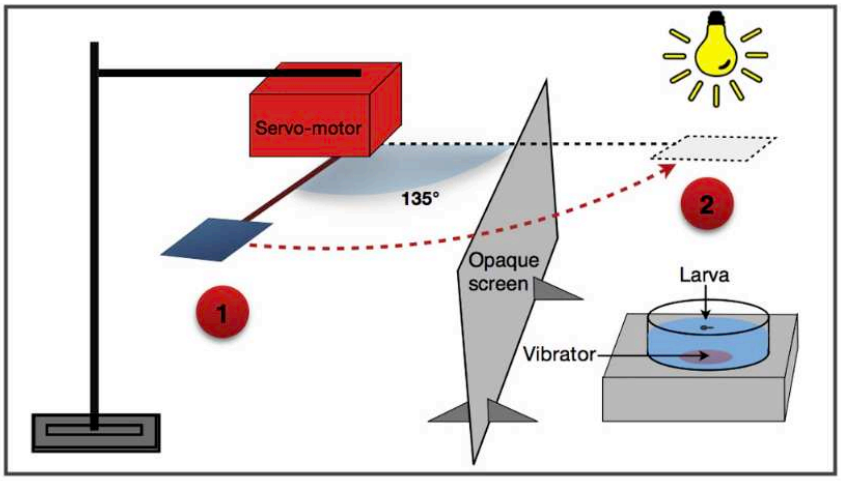
« *Larvae exposed frequently to shadows gradually fail to respond to them. A dish containing about thirty three-day old larvae was shaded by passing an object over it once every minute, the object being passed as nearly as possible in the same way and with the same degree of speed. At the first trial all (about twenty-five) which were at the surface went down. At the second shading about fifteen went down, leaving nine at the surface. In subsequent trials the number remaining at the surface gradually decreased. From the twelfth to the sixteenth trials none descended; in the seventeenth, six went down, after which numerous subsequent trials produced no response* ». (Holmes 1911).

Malgré le manque de données quantitatives dans ses expériences, Holmes observait déjà il y a plus d'un siècle une diminution graduelle de la réponse comportementale de fuite des larves vis-à-vis d'un stimulus, une ombre créée par un objet volant au-dessus de l'enceinte où étaient situées les larves. En 1946, Thomas montre que ce comportement de fuite dépend de **l'âge des individus** et de la **densité des larves** présentes chez *Culex fatigans,* puis Leftwich nomme ce comportement « le réflexe de submersion », avant que Mellanby le renomme « réaction d'alarme » et le compare entre *Culex*, *Aedes* et *Anopheles* (Mellanby 1958).

En 1978, Kasap utilise un kymographe, appareil qui inscrit graphiquement des positions spatiales au cours du temps, en se servant de la broche rotative de cet appareil où il fixe un disque. Il place une lampe à 35 cm au-dessus du disque et place 6 individus dans un bécher de 6 cm de hauteur. Toutes les 5 min, le disque passe par-dessus l'enceinte et Kasap note le nombre d'individus qui plongent. Ce stimulus est répété six fois avec un entrainement le matin et un l'après-midi. Kasap trouve globalement une

diminution de la réponse au cours du temps, avec des différences entre espèces. *Aedes* présente une réponse comportementale plus rapide et les larves sont plus actives que *Culex* et *Anopheles*. Kasap émet l'hypothèse **qu'il est plus avantageux de fuir rapidement si on est plus rapide que notre prédateur, alors qu'il vaut mieux rester immobile si on est moins rapide**. Il note enfin un important effet de l'âge : il y a une diminution graduelle de la réponse avec l'âge, qu'il explique notamment par une augmentation de la consommation en oxygène requise pour se déplacer.

Presque 40 ans après Kasap, Baglan et al. (2017) utilisent un dispositif semblable tout en le modernisant (Figure 18). Ici, les larves sont disposées individuellement dans des boites de Petri plus petites, 1.2 cm de hauteur, et chaque larve est soumise à un stimulus aversif formé par un morceau de carton rectangulaire. 10 réplicats sont observés par un expérimentateur, en série, avec également 5 min d'intervalle entre essais pour un total de 10 essais. Les auteurs rajoutent deux contrôles au groupe entrainé, et comparent la réponse au nouvel essai (test) qui sera effectué pour chaque groupe après l'entrainement. Dans le premier contrôle, les larves ne sont pas entrainées, elles sont au repos dans l'enceinte pendant toute la durée de l'entrainement. Ce contrôle permet de vérifier que le contexte du dispositif expérimental ne perturbe pas la réponse comportementale des larves. Pour le deuxième contrôle, les larves sont entrainées et perturbées après l'entrainement : un vibrateur, situé sous l'enceinte, est actionné 5 min après l'entrainement et 5 min avant le test. Ce dispositif permet alors de valider le 2ème et le 8ème critère établi par Rankin et al. (2009), c'est-à-dire la réponse spontanée et la déshabituation. Il permet donc **d'exclure la fatigue et l'adaptation sensorielle chez *Aedes aegpyti***. Les auteurs vont également utiliser ce dispositif en 2018 et en 2019 pour étudier l'effet du glyphosate sur l'apprentissage des larves *Aedes aegpyti* ainsi que la spécificité du stimulus. Ils ont montré d'une part que **le glyphosate altère l'apprentissage des larves à des doses sous-létales**, et d'autre part que **l'habituation du stimulus est spécifique au contexte** via le motif du stimulus visuel (Baglan et al. 2018; Pientrantuono 2021).



	Training														Disturbance	Test
Training group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	-	+
Training + Disturbance group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	+	+
Untrainded group	*rest*															-	+

0 min ⟷ 75 min ⟷ 80 min

Figure 18: A) Dispositif. Une larve est placée dans la boite de Petri. Un moteur actionne la rotation du carton qui va créer un contraste lumineux au-dessus de la boite de Petri. Un vibrateur est actionné manuellement sous la boite de Petri. L'écran opaque sépare visuellement la larve des autres réplicats. B) Trois groupes utilisés : le groupe entrainé et testé, le groupe entrainé, perturbé et testé, le groupe non-entrainé et testé. D'après Baglan et al. (2017).

Le dispositif présenté dans ces études permet de comparer des réponses comportementales selon plusieurs modalités d'entrainements. Il permet également d'observer une réponse individuelle, de faire varier le contexte, et d'appliquer de manière automatisée un stimulus visuel. Cependant, certains points pourraient être améliorés. Par exemple, **l'observation visuelle estime de manière approximative la réponse comportementale des larves**. En effet, le mouvant du bras dure 0,4 s, ce qui laisse peu de temps pour observer le comportement des larves vis-à-vis du stimulus. Avec cette hauteur de boite de Petri, le comportement observé est le gigotement, qui est similaire au comportement de locomotion très fréquent chez les larves *Aedes aegypti*. Ainsi, déterminer si la larve réagit ou non de manière binaire à un stimulus de courte durée peut être ambivalent via la résolution visuelle de l'œil humain (Dell et al. 2014). La répétition de l'observation dix fois par minutes, et ceci douze fois pendant 1h 30 min est

également un exercice qui demande de l'énergie, et l'observation visuelle inclut les **biais inter et intra-observateurs**. D'une part, le seuil individuel de détection d'une réponse est différent, et d'autre part, chaque expérimentateur est soumis à des biais (Bohlen et al. 2014). En effet, il peut inconsciemment modifier son seuil d'observation au cours du temps, ou attribuer une évaluation plutôt positive (l'individu répond) en début d'expérience et plutôt négative (l'individu ne répond plus) en fin d'expérience s'il a des prédictions à priori (Rosenthal et Rubin 1978). Enfin, le manque de **mise en aveugle** lors des comparaisons entre traitements peut influencer les prédictions à priori, et le manque de contrôle de certains paramètres (ex : intensité lumineuse ou taille de l'ombre projetée) peut également influencer les résultats (Burghardt et al. 2012). En 2021, l'entreprise Zantiks a proposé un dispositif qui utilise des vibrations mécaniques à fréquences précises et observe le comportement des larves *Culex*. Le preprint mis en ligne indique que la réponse comportementale des larves observée ici est verticale, et quantifiée via un enregistrement vidéo et un logiciel de tracking (Harrison et Budenberg 2021).

La larve de moustique représente un modèle avantageux et au grand potentiel pour des études en écotoxicologie. Via l'habituation, ses capacités cognitives peuvent être explorées, mais les paradigmes expérimentaux et les dispositifs doivent être adaptés afin de produire des données quantitatives et de haut débit, d'être objectifs et d'être pertinents en lien avec les variables environnementales. **Dans cette thèse, je me suis inspiré d'anciens protocoles expérimentaux afin de mettre en place un nouveau dispositif qui répond aux enjeux mentionnés ci-dessus. Celui-ci comprend un paradigme d'apprentissage par habituation chez la larve de moustique, et tente d'explorer l'effet de l'environnement sur les propriétés cognitives des larves.**

Objectifs de la thèse

Est-ce que la cognition des larves de moustique pourrait être un bon indicateur biologique de la qualité des écosystèmes d'eau douce ?

Notre hypothèse générale est que toute perturbation de l'environnement impacte les organismes. En particulier, la capacité à apprendre et à mémoriser des informations représentent des mécanismes complexes qui nécessitent un traitement de cette information et qui sont en première ligne pour être impactée par des modifications environnementales, particulièrement la présence de polluants sous-létaux et en co-occurrence. En mesurant l'effet de l'environnement sur la cognition des larves de moustique, nous tentons d'avoir une mesure indirecte de la qualité de l'écosystème dans lequel se développent ces larves.

En comparant les performances des larves de trois espèces de moustique, nous avons tenté :

- D'adapter l'expérience de Baglan et al. (2017) chez l'espèce *Aedes aegypti*, afin de tester simultanément dix individus via un dispositif automatisé et paramétrable

- De valider l'apprentissage par habituation chez les genres *Culex* et *Anopheles*

- D'explorer leurs capacités de mémorisation

- D'observer l'effet de polluants à doses sous-létales sur leurs capacités cognitives

- De comparer les performances d'individus provenant de milieux naturels soumis à différentes sources de pollution

Plan de la thèse

Dans le **Chapitre 2**, je présente un nouveau dispositif automatisé que nous avons développé durant ma première année de thèse et qui nous a permis d'explorer l'apprentissage par habituation chez trois espèces de moustique. Dans le **Chapitre 3**, j'explore les limites de la capacité de mémorisation chez *Aedes Aegypti*. Dans le **Chapitre 4**, je compare les performances d'apprentissage, de mémorisation et de locomotion chez *Aedes aegypti*, exposées à des polluants à doses sous-létales. Dans le **Chapitre 5**, je compare les performances d'apprentissage, de mémorisation et de réactivité visuelle chez les genres *Culex* et *Anopheles*, provenant d'étangs de la région Centre-Val de Loire. Enfin, dans le **Chapitre 6**, je discute de l'ensemble de nos résultats afin de cerner les avantages et inconvénients de notre méthodologie, les implications en écotoxicologie et les perspectives concernant l'utilisation de la cognition des larves de moustique comme indicateur des écosystèmes.

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Chapitre 2 : Évaluer l'apprentissage des larves de moustique par détection vidéo

Pour moi, faire de la recherche, c'est comme jouer au backgammon. Je ne sais pas jouer au backgammon.

Martin **Dessart** (2023)

Les larves de moustique possèdent un **comportement de fuite** qui peut être étudié dans le cadre **d'apprentissage**. Au repos, les larves de moustique sont attachées à la surface de l'eau par un siphon respiratoire. Si un stimulus aversif survient, comme une ombre en mouvement, les larves présentent un comportement stéréotypé de fuite en se détachant de la surface et en plongeant le long de la colonne d'eau. Dans ce chapitre, nous décrivons un **système automatisé** basé sur le suivi vidéo et l'extraction de données quantitatives des mouvements des larves à l'échelle individuelle. Dans un premier temps, nous avons validé notre protocole en examinant à nouveau **l'habituation** chez les larves de l'espèce ***Aedes aegypti***, élevées en laboratoire. Nous avons également évalué pour la première fois **l'habituation** chez les genres ***Culex*** et ***Anopheles***, grâce à la collection d'individus sur le terrain. Enfin, nous avons pu caractériser **l'activité motrice** des individus grâce à l'analyses des données fournis par notre dispositif. Le système et les algorithmes présentés ici peuvent facilement être adaptés à différents contextes expérimentaux.

Ce chapitre a fait l'objet d'une publication dans : **Journal of Insect Physiology** et figure en annexe de ce manuscrit de thèse.

Dessart M, Piñeirúa M, Lazzari C, Guerrieri F (2023) Assessing learning in mosquito larvae using video-tracking. Journal of Insect Physiology 149:104535. https://doi.org/10.1016/j.jinsphys.2023.104535

Assessing learning in mosquito larvae using video-tracking

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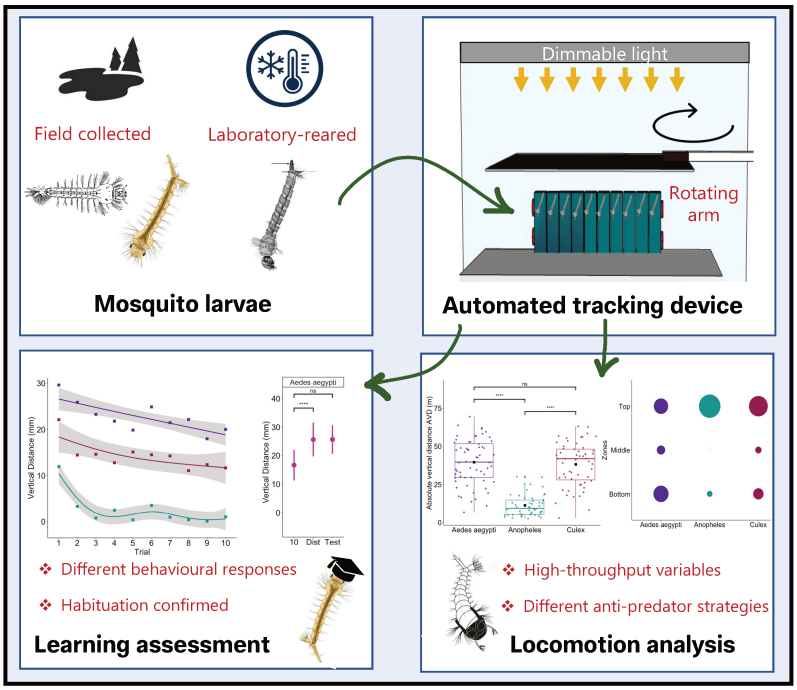
Keywords: habituation, non-associative learning, *Aedes, Culex, Anopheles*

Highlights

1. An automated apparatus to study habituation in mosquito larvae is presented.
2. Full control of training and testing parameters was easily practicable.
3. Different behavioural reactions could be video-tracked, quantified and recorded.
4. Habituation to a visual stimulus was characterised in *Aedes, Culex* and *Anopheles*.
5. Differences in learning performances across mosquito species could be unravelled.

Graphical abstract



Abstract

Mosquito larvae display a stereotyped escape response when they rest attached to the water surface. It consists in detaching from the surface and diving, to return to the surface after a brief time. It has been shown that this response can be evoked several times, by repeatedly presenting a moving shadow. Diving triggered by a potential danger revealed as a simple bioassay for investigating behavioural responses in mosquito larvae, in particular their ability to learn. In the present work, we describe an automated system, based on video-tracking individuals, and extracting quantitative data of their movements. We validated our system, by reinvestigating the habituation response of larvae of *Aedes aegypti* reared in the laboratory, and providing original data on field-collected larvae of genera *Culex* and *Anopheles*. Habituation could be demonstrated to occur in all the species, even though it was not possible to induce dishabituation in *Culex* and *Anopheles* mosquitoes. In addition to non-associative learning, we characterised motor activity in the studied species, thanks to the possibility offered by the tracking system to extract multiple variables. The here-described system and algorithms can be easily adapted to multiple experimental situations and variables of interest.

Introduction

Adapting an individual's behaviour on the basis of its own experience (i.e. learning) and remember past experiences (i.e. memory) are crucial for animal survival and decision making (Evans et al. 2019).

Habituation is a particular form of non-associative learning (Thomas, 1949; Leftwich 1954) which consists in no longer reacting to stimuli that trigger behavioural response in naïve animals and turned out to be innocuous (See Rankin et al 2009 for review). For example, when a moving object casts its shadow over the water surface, mosquito larvae dive escaping from a potential danger (Holmes 1911). After several passages of an innocuous shadow, larvae stop responding, even though they still detect it and they are able to dive. The individuals do not perceive the stimulus as a potential danger anymore, i.e. the larvae become habituated to its presence (Baglan et al., 2017).

Habituation protocols in mosquito larvae have revealed to be reliable bioassays, not only for testing cognitive abilities of these insects (e.g. Baglan et al. 2017; Pientrantuono et al. 2021), but also as a proxy for evaluating the impact on living creatures of chemical pollutants in water (Baglan et al. 2018). In a typical experiment, an observer records whether or not individual larvae move during the controlled passage of a moving shadow, attributing a score of 0 or 1. The shadow is presented at regular intervals, until the insects stop responding. Specific tests follow, in order to assess whether the behavioural change is either due to learning or to other physiological processes, such as sensory adaptation or motor fatigue (for a review, see Rankin et al. 2009).

In this work, we present an original system allowing the automated quantification of different components of the response of mosquito larvae to a potential danger. Our tracking software allows accurately measuring individual response, and calculating different metrics associated with diverse components of the behavioural response, minimising experimental biases.

The system allows training and testing several individuals in parallel in a single session, saving time, increasing the number of replicates, and obtaining accurate quantitative data on different behavioural variables. Training parameters as intensity and

duration of the stimulus, inter-trial interval, interval between training and test can be precisely adjusted by the experimenter. We started by testing and validating our system and the experimental protocol for habituation experiments in a reference species (i.e. *Aedes aegypti*) and then we compared the responses among laboratory and field-collected mosquito larvae of other species.

Material and methods

1. Animals

Aedes aegypti (Bora strain) were obtained from eggs provided by the INFRAVEC2 European project and reared at VECTOPOLE-IRD (Montpellier, France). The eggs were placed in small plastic containers filled with dechlorinated tap water and fed *ad libitum* with shrimp food (JBL Novo Prawn, Neuhofen, Germany). The larvae were maintained in a climate-controlled room at 25°C ± 2°C, under 12h:12h light:dark illumination regime (lights on at 8:00).

Culex and *Anopheles* larvae were collected in two natural habitats located in the department of Indre et Loire, France. The first site was a 10-ha basin (*Étang de l' Archevêque*) located in the Loire Valley (47°31'N, 0°51'E), and the second was a pond situated in an urban garden in the city of Tours (47°23'N, 0°41'E). At each site, captures were carried out over a sampling area of approximately 1 m^2 by scooping the surface with a 1-litre round recipient (O'Malley 1995), until at least 100 individuals were collected. Sampled individuals were kept in water from their natural habitat during the journey back to the laboratory and then gently transferred to 750 ml polypropylene plastic containers and reared similarly as *Ae. aegypti* larvae. Before experiments, individuals were kept undisturbed between 24 h and 48 h under laboratory conditions (the same for all species). Fourth-instar larvae were used in all the experiments.

All animals were collected, reared and manipulated according to ethics regulations applied in the European Union.

2. Identification

For *Culex* and *Anopheles*, the morphological identification of individuals was performed under a stereomicroscope, with the aid of the MosKeyTool database (Gunay et al. 2018). An initial identification was conducted after the experiment to assess the genus at larval stage. Individuals were kept until emergence, then adults were identified a second time to determine sex and confirm genus, using the same key.

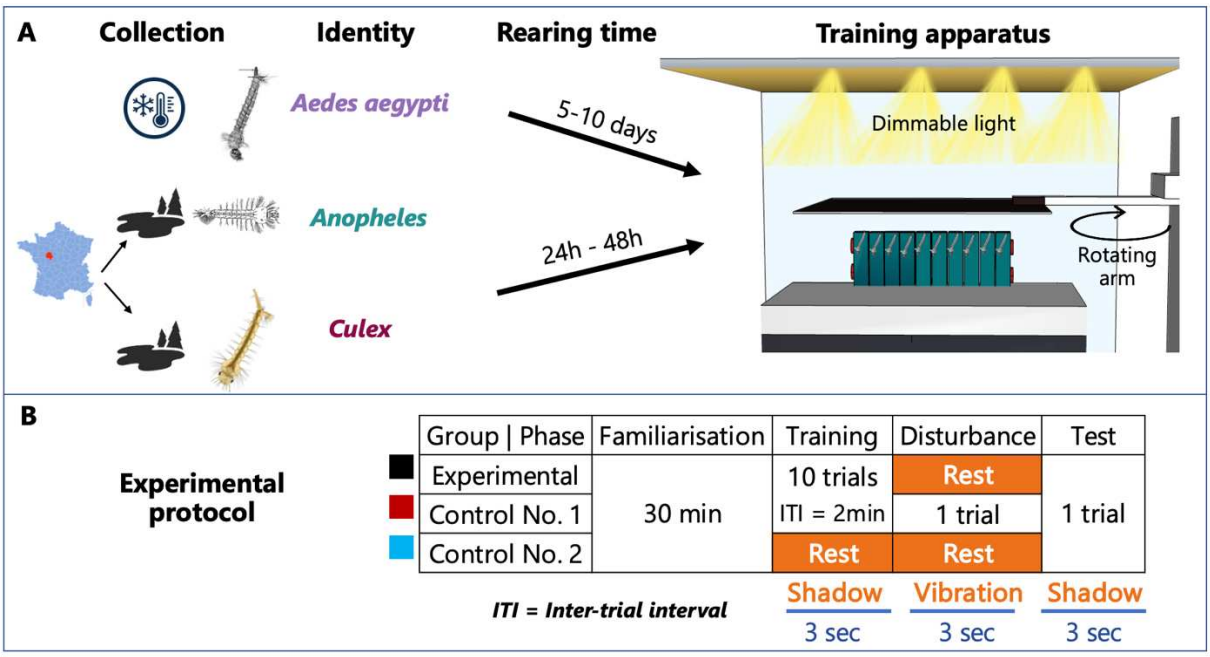


Figure 1: Experimental protocol. A) We collected *Culex* and *Anopheles* larvae in two ponds located in Indre-et-Loire, Région Centre-Val de Loire, whilst *Aedes aegypti* larvae were reared in the laboratory. We trained individuals of the 4th larval stage using an automated device. B) We quantified the responses of three groups: 1) trained and not disturbed (Experimental); 2) trained and disturbed (*Control No. 1*); 3) untrained (*Control No.* 2).

3. Experimental setup

The experimental apparatus (Figure 1) consisted of two light sources, a camera, and a servo mechanism, which controlled the projection of the shadow of a square cardboard (*shadow*) above twelve 1.5 ml spectrophotometer plastic cuvettes (Z187992-1PAK, Sigma-Aldrich, Germany) where the larvae had been individually placed. One light source consisted of two LED panels (30 cm x 30 cm), located above the cuvettes. The second light source was an infrared backlight (880 nm) placed behind the cuvettes. In front of the cuvettes, a camera (acA 1300 – 60gc, Basler, Germany) equipped with a high-pass infrared filter (RG 850 Filter - 40.5 mm, Heliopan, US) recorded the experiments.

In order to exclude unwanted vibrations, cuvettes stood on a 2 cm-thick polystyrene plate resting over acoustic foam. The lateral faces of each cuvette were covered with opaque white tape, in order to avoid mutual visual influence. A water-filled cuvette without larvae was placed at the left of the 1st cuvette and another one at the right of the 10th cuvette, to minimise any effect of cuvette position.

Two stimuli of different modality could be presented. The first (visual) was the shadow projected by a black cardboard square (16 cm side) attached to a wooden stick and fixed to a servomotor controlled by an Arduino Uno board (http://www.arduino.cc). During a stimulation, the stick turned 100° and returned back to the resting position (Figure 1). The second stimulus (mechanical) was the vibration produced by a set of 4 identical vibrators (3.3 V, 100 mA; 11000 rpm; 10 mm diameter; 2.7 mm height, Radio Spares, France), controlled by the same Arduino Uno board. Two vibrators were placed on the outer side of the left end and the other two at the right end, i.e., on the side of the unoccupied cuvettes. The Arduino board was remotely controlled by a computer, which was also connected to the camera.

Preliminary tests revealed differences in the responsiveness of the larvae belonging to the different species. For this reason, a series of experiences were run in order to establish the appropriate parameters for testing each species.

For *Ae. aegypti* light intensity was set at 900 µW.cm^{-2} ± 100 µW.cm^{-2} (International Light Technology radiometer). The distance between the top of the cuvettes and the rotating arm was established in 5 ± 0.2 cm and the stimulus duration fixed at 3 s at an angular velocity of 0.067°/ms.

For *Culex* and *Anopheles* larvae, we increased the light intensity to 1500 µW.cm^{-2} ± 100 µW.cm^{-2} and placed the card closer to the top of the cuvette (0.3 ± 0.1 cm). We also increased the arm rotation to 7.5 °/ms and added 1.5 s of delay in the stimulus position, to keep the total stimulus duration at 3 s. The goal was ensuring that most of the larvae would react.

4. Experimental conditions

All experiments were performed in a room kept at the rearing temperature. Larvae were carefully removed from the rearing container and placed individually in the cuvettes filled with dechlorinated tap water. Larvae were left undisturbed during 30 minutes for

familiarisation before starting the experiment. Under these conditions, we established one experimental group and two control groups. The procedure consisted of three phases: *training or rest*, *disturbance or rest* and *test* (Figure 1).

The *Experimental* group was set to assess the decrease in response induced by the repeated presentation of the visual stimulus (*training*). Larvae were confronted with 10 passages of the shadow (i.e. *trials*), spaced by inter-trial intervals (ITI) of 2 minutes. After the 10th presentation of the stimulus, larvae remained undisturbed during 4 minutes, before the final presentation of the shadow; i.e., the *test* phase.

Control No. 1 (disturbance) was set to assess dishabituation (Rankin et al. 2009). Larvae were exposed to 10 stimuli, similarly to the experimental group (*training*). Two minutes after the 10th stimulus, a vibration was applied (*disturbance*). This disturbance was followed by 2 minutes ITI and the final presentation of the shadow (*test*).

Control No. 2 (untrained), after familiarisation, larvae remained confined in the cuvettes during 22 minutes without receiving any stimulation. Subsequently, the visual stimulus was presented to the larvae only in the *test* phase.

After the end of each experiment, larvae were gently removed from the cuvettes and individually kept in identified Petri dishes (3-cm diameter) during 24 h. Those that emerged as adults during this time could have been pharate pupae during the experiment and consequently excluded from the analyses.

5. Video analyses

Each experiment was recorded and two sets of videos (resolution 640x480 px, 25 fps) were produced (Figure 2, step 1). The first one consisted of sequences of the last 5 minutes of familiarisation. The second set consisted of 26 minutes videos of the three phases of each experiment (i.e., *training*, *disturbance* and *test*). The videos were analysed using a modified version of the image-based freeware Tracktor (Sridhar et al. 2019).

The tracking software was based on a contour identification algorithm relying on the contrast between the larvae and the background. (Figure 2, step 2 and 3). During the video analysis, the position and contour area of each larva were measured while keeping identity (Figure 2, step 4). At the end of the video analysis measurements were exported to a *.csv* file.

The tracking results were analysed using R version 4.1.1 (2021-08-10) (https://cran.r-project.org/). Horizontal coordinates were used to verify that larvae identities were respected (each larva detected inside a given cuvette, Figure 2, step 5). To check the performance of the tracking, the detection rate was calculated by taking the maximum frame length available on the video and multiplying it by the number of individuals. This rate was compared to the actual number of frames identified by the tracking software and we ensured that at least 80% of the data present (Figure 2, step 6). The vertical position data were smoothed using the *rollmean* function in the *zoo* package (Zeileis and Grothendieck, 2005) with a 10-frame window (Figure 2, step 7).

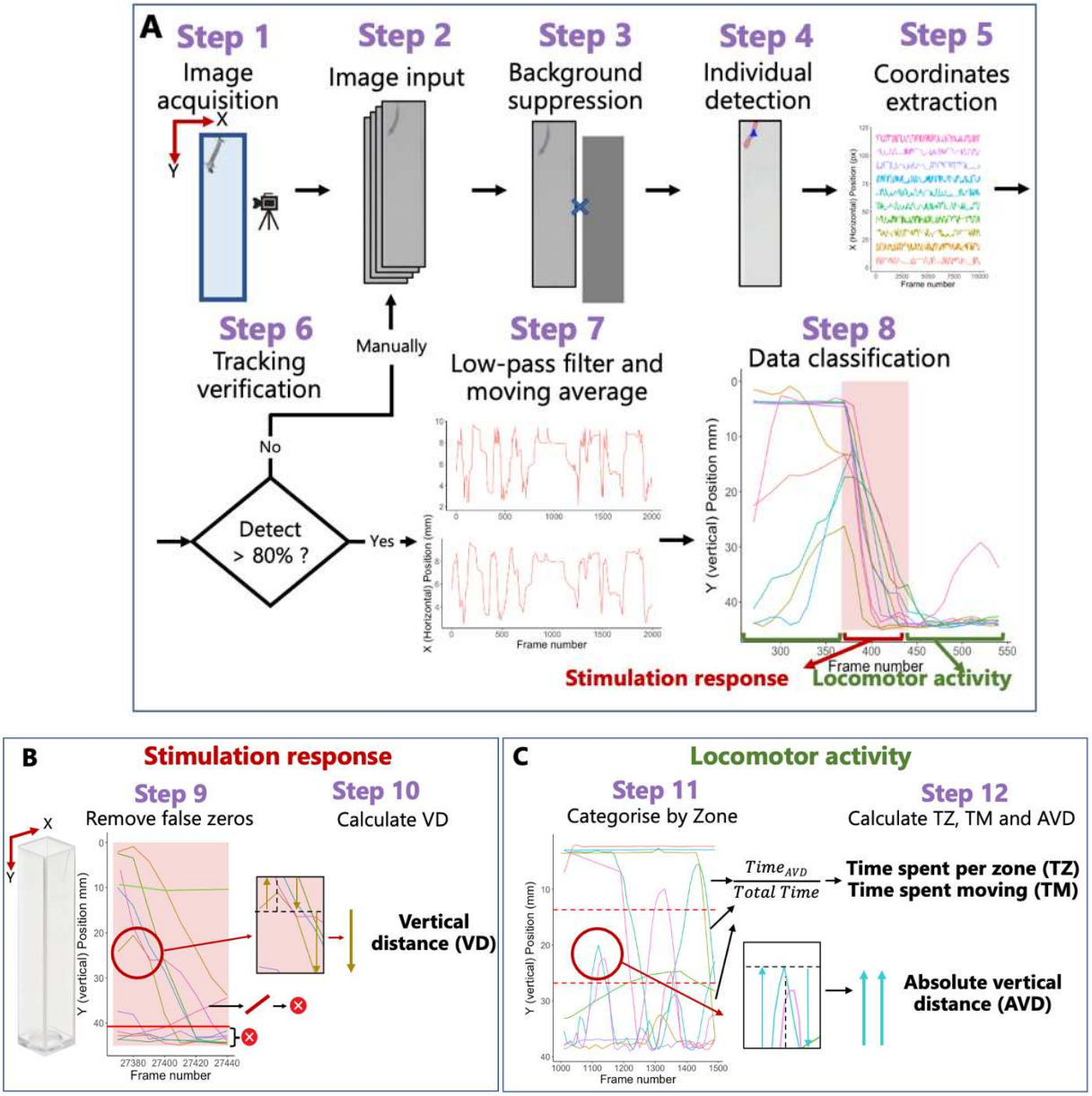


Figure 2: recording and quantifying individual behaviour. A) Flowchart of data acquisition and treatment. B) Stimulation response (SR) was analysed using two metrics. Performance Index (PI) was binary and calculated following the trajectory direction of each individual for each trial. Vertical distance (VD) was quantitative and calculated as the relative sum of the distance travelled in the vertical direction. C) Locomotor activity (LA) was analysed using three metrics. Time spent per zone (TZ) was a proportion of time spend in one of the 3 zones delimited. Time spent moving (TM) was a proportion of time where the Absolute vertical distance was above a threshold of 1mm/sec. Absolute vertical distance (AVD) was quantitative and calculated as the absolute sum of the distance travelled in the vertical direction.

6. Data classification and analysis

From each dataset and each trial, we defined and extracted the 3 seconds trial period as the duration of the stimulus appearance over the individuals (Figure 2, step 8). Therefore, we could extract 8 successive positions for each individual and for each trial that were classified as Stimulation Response dataset (Figure 2).

No vertical displacement could be observed in the larvae that were at the bottom of the cuvette at the beginning of a trial. Therefore, we excluded the response of individuals whose vertical position at the start of a trial was higher than 9/10th of the cuvette length (i.e. close to the bottom) (Figure 2, step 9).

Vertical distance (VD) was the response variable and corresponded to the escape response, starting from 0 at the top of the cuvette, and increasing, when larvae dived along the water column (Figure 2, step 10).

We also defined a binary Performance Index (PI) (Figure 2, step 10) as:

$$PI = \begin{cases} 0 \; for VD < \dfrac{max(VD)}{5} \\ 1 \; otherwise \end{cases}$$

For *Culex* and *Anopheles* species, some individuals rested completely motionless during one trial. For this trial, the value given for their displacement was therefore counted as 0 mm for VD and for PI. When one individual was completely immobile during the acclimation and the training period, it was removed from the database (8.5% for *Anopheles*, 0% for others). A total of 246 individuals were retained for the analysis (Table 1).

	Aedes aegypti				*Culex*				*Anopheles*				All
	Experimental	**Control n°1**	**Control n°2**	**Total**	**Experimental**	**Control n°1**	**Control n°2**	**Total**	**Experimental**	**Control n°1**	**Control n°2**	**Total**	**Total**
Individuals trained	30	30	30	90	26	29	27	82	22	28	24	74	246
Trials per individuals	360	360	30	750	310	347	27	684	191	313	24	528	1962
Trials filtered by position	274	268	24	566	271	312	27	610	189	301	23	513	1689
% Trials removed	23.9%	25.6%	20.0%	24.5%	12.6%	10.1%	0.0%	10.8%	1.0%	3.8%	4.2%	2.8%	13.9%
Trials filtered by going up	268	263	24	555	261	290	26	577	184	293	23	500	1632
% Trials removed	2.2%	1.9%	0.0%	1.9%	3.7%	7.1%	3.7%	5.4%	2.6%	2.7%	0.0%	2.5%	3.4%
Total % Trials removed	25.6%	26.9%	20.0%	**26.0%**	15.8%	16.4%	3.7%	**15.6%**	3.7%	6.4%	4.2%	**5.3%**	**16.8%**

Table 1: Summary of the filtering steps. For each species, 22 to 30 individuals were trained. When the individual's position was close to the bottom, the response to the trial was removed, accounting for a total of 13.9% of trials removed. The trajectory of an individual moving upwards during a stimulation was rare, representing only 3.4% of trials.

As individual positions were recorded throughout the whole experiments, we also extracted data during the 9 inter-trial intervals (ITIs) and analysed the locomotor activity during these periods (Figure 2). We first calculated the Absolute Vertical Distance (AVD) travelled by individuals by summing the AVD for all ITIs per individual during the training session (Figure 2, step 11). We also ranked data by ITI and compared the AVD per ITI for each species. The AVD was then averaged per second and calculated for each individual to compare the individual average speed during the ITIs. The maximum speed of each individual was also compared in the same way.

We divided the cuvette in three equal zones (top, middle, bottom, Figure 2, step 12) and calculated the time spent per zone. We used these zones to develop another metric corresponding to the diving events. If an individual crossed two successive zones on the way in and out, we considered this to be a diving event. To analyse if an individual was moving or not based on a dichotomial rule, we also confronted the individual AVD to a threshold of 1 mm per second and classified the resulting data in Time spent moving (Figure 2, step 12). Prior to any training, individuals were recorded for 30 minutes during the familiarisation period. To highlight the effect of the stimulation on individual activity, we analysed the last 5 minutes of familiarisation and compared them to the ITI periods. Finally, using contour tracking data, we were able to compare the maximum individual surface detected by the tracking between species, i.e., the area representing each individual in pixel.

Statistical analyses

1. Data classification and filtering

For the three species, we verified whether responses to stimulation were trial-specific (i.e. increased or decreased) by applying a Chi-square goodness of fit test. The rationale behind this verification was to exclude that larvae could have changed their position before the release of the stimulus over the course of the training, then biasing the output of the filtering.

2. Comparison across species

For the three mosquitoes, we used a Generalised Additive Model to explore different response curves for each variable and their effect on the generalised cross-validation unbiased risk estimator (GCV-UBRE) (Zuur et al. 2009). We defined models of increasing complexity and different smoothing functions and compared them using the GCV-UBRE of the *mgcv* package (Wood 2017).

To compare locomotor activity between species, we used linear mix-effects models. These models were used for the comparison of AVD (m), average speed (mm/s) and maximum speed (mm/s), time spent per zone (%) and time spent moving (%), number of diving event and maximum surface (mm^2). We chose species as a fixed factor and individual identity as a random factor. Post-hoc comparisons were analysed using the *emmeans* function from the *emmeans* package (Russell 2021).

3. Learning performance

To assess the learning performances of the different groups of larvae, we compared Vertical Distance and Performance Index. VD was evaluated by means of a linear mix-effects model and PI of a GLMER with a logit link and a binomial distribution, with trial number and group as fixed factors, and individual identity as random factor to account for repeated measurements. The group factor served to evaluate eventual effects due to contexts across groups of larvae trained in a similar way. As the interaction between trial and group was never significant, we dropped the interaction from the model.

4. Test phase

Test responses were analysed by running a linear mix-effects model for VD and a GLMER for PI with a logit link and a binomial distribution. VD and PI were chosen as the response variables; group as fixed factor, and individual identity as random factor.

5. Dishabituation

To assess dishabituation, we compared VD and PI at the tenth trial with the response at the *disturbance* and at the *test* phase. VD was compared by using a linear mix-effects model and PI by means of GLMER, similarly as in the section Learning performance.

6. Dataset and analysis code repository

The version of the tracking software used to characterise individual behaviour and the R code used to analyse data and display the figures were made available online at: https://github.com/martindessart/Tracking-system.

Results

1. Identification

Larvae and adults could be identified at the genus level. For *Anopheles,* we were able to evaluate the sex of 25 individuals out of the 74 total individuals trained, of which 13 were identified as females and 12 as males. For *Culex,* 40 individuals were identified as females and 24 as males.

2. Data classification and filtering

At 25 frames per seconds, a 22-minutes recording corresponded to a total of 33 000 frames. Our tracking algorithm, adapted from the open source software Tracktor (Sridhar et al. 2019) had a tracking time of less than 20 minutes. For comparison, the zebrafish video from ToxTrac software (Rodriguez et al. 2018), with a resolution of 32 frame per second and 15 000 frames, had a tracking time of 9 minutes 43 seconds using Tracktor software (Sridhar et al. 2019). Regarding accuracy, the total percentage of

detection rate was 92.54% for 22 records. The highest detection rate was 99.97% and the lowest 84.84%. For each experiment, we performed a calibration by zooming in on the cuvettes with our camera and applying the function "Automatic image adjustment" from Basler Pylon5 software (http://baslerweb.com). Provided that each cuvette was physically separated from the others, a handmade function on R software using the total horizontal distance (*x*-coordinate) divided by the number of individuals allowed us to identify all individuals for all videos. Finally, for each recording, we manually selected the square outline of the 10 cuvettes. Then, for each species, we took the mean of the maximum distance in pixels for each record and converted the pixel unit to millimetres. The mean distance was: Mean 401.4 px, SD = ±9.01 px.

Our tracking system was able to discriminate individuals based on their vertical position at the start of each stimulation. When an individual was above a threshold of 9/10th of the total length of the cuvette, the filtering step (Figure 2, step 9) eliminated an average of 13.9% of the trials for all species (Table 1). This percentage depended on the species, with 24.5% for *Ae. aegypti,* 10.8% for *Culex* and 3.0% for *Anopheles*. Furthermore, the Performance Index calculation detected and eliminated an additional 3.3% of responses to trials where the individual VD was greater than 1/5th of the maximum VD. This was more pronounced for *Culex* (5.4% of removed trials) than for *Anopheles* (2.4%) and *Ae. aegypti* (1.9%). The overall process resulted in 1632 stimulation responses for 246 individuals. To determine whether the trials deleted in these two successive steps were trial-specific, we confronted this hypothesis using a Chi-square goodness of fit test. The deleted trials were not specific to a trial number for the three species: *Ae. aegypti*: χ^2 = 10.81, df = 11, P = 0.459; *Anopheles:* χ^2 = 8.11, df = 11, P = 0.703; *Culex*: χ^2 = 15.59, df = 11, P = 0.157.

3. General comparison across species

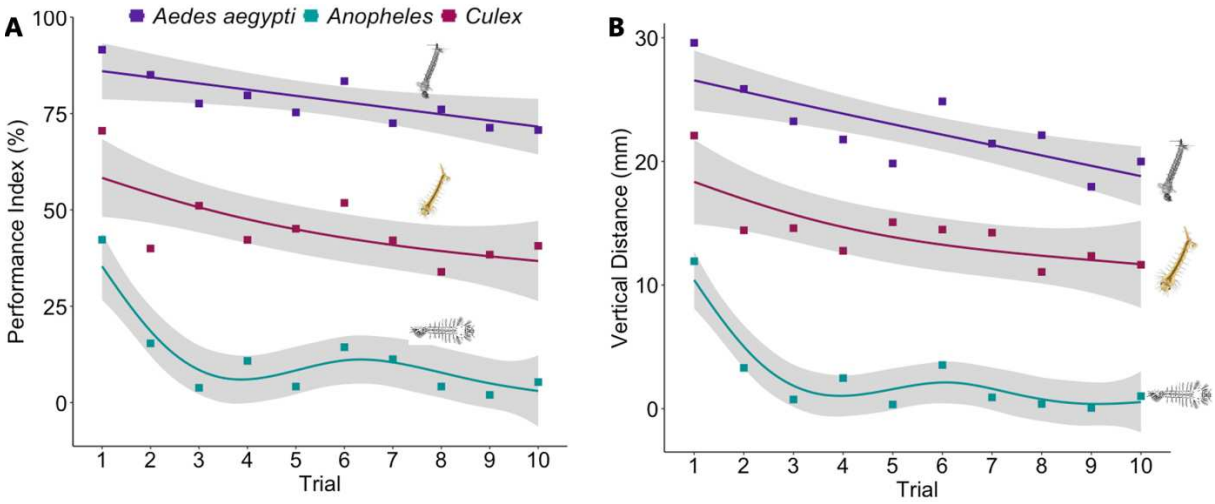


Figure 3: behavioural response to the repetition of an aversive stimulus. A) Performance Index of individuals responding to an aversive stimulus in each trial. B) Vertical distance in millimetre travelled by individuals responding to an aversive stimulus during the 1st to the 10th trial. Smoothing lines indicate the best fitted GAM model. Grey shades indicate 95% confidence interval. Points indicate mean values.

The response of the larvae decreased with the consecutive passage of the shadow. In other words, the escape response was less intense over the course of the ten trials, both for PI and VD (Figure 3A, B). Concerning PI, a difference in the number of responsive larvae (PI = 1) was observed across species, being *Ae. aegypti* the most responsive and *Anopheles* the least. All three decreased over the trials at similar rates, but curves ran parallels at different levels.

To describe the variation in VD, the best smoothing function was the P-spline, as it is based on equally spaced knots (Wood 2017). Plotting the mean distance (mm) against the number of trials for the three species revealed different responses (Figure 3B). *Ae. aegypti* responded strongly to the stimulus (Mean = 22.69, SEM = ±0.63), *Culex* was weaker than *Ae. aegypti* (Mean = 16.09, SEM = ±1.11) and *Anopheles* responded the least (Mean = 6.50, SEM = ±0.1) (Figure 3A, B). *Anopheles* also decreased their response more steeply than *Ae. aegypti* and *Culex* (Figure 3A, B).

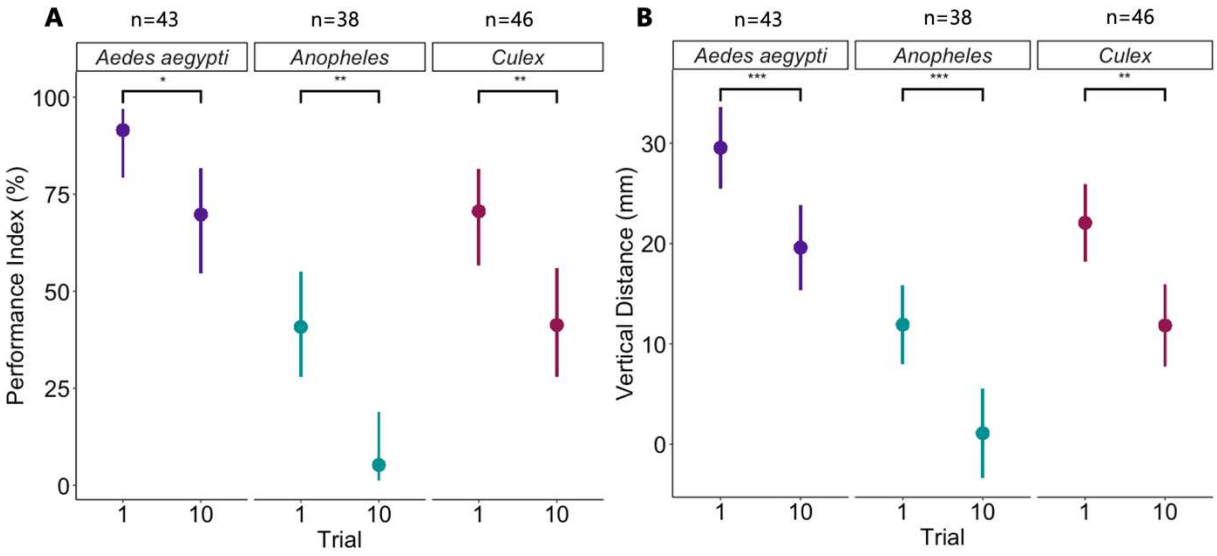


Figure 4: Learning performance. A) Performance Index of individuals responding to an aversive stimulus in the 1st trial and in the 10th trial. B) Vertical distance travelled by individuals responding to an aversive stimulus in the 1st trial and in the 10th trial. Points indicate mean values and bars indicate +- 95% confidence intervals. NS, not significant; *P<0.05, **P<0.01, ***P<0.001.

Concerning spontaneous locomotor activity, *Ae. aegypti* and *Culex* moved significantly more than *Anopheles* (P < 0.0001 in both cases) but did not differ from each other (P= 0.758, Supplementary Figure 1A). Regarding within-trial differences, the three species did not show significant differences among trials (*Ae. aegypti*: df = 8, P = 0.990, *Culex:* df = 8, P = 0.999, *Anopheles*: df = 8, P = 0.100, Supplementary Figure 1B). Regarding the average speed, while *Ae. aegypti* and *Culex* were significantly faster than *Anopheles* (P < 0.0001 in both cases, Supplementary Figure 2A), the latter reached higher maximum speed than *Ae. aegypti* and *Culex* (P < 0.0001 in both cases). *Culex* had a higher maximum speed than *Ae. aegypti* (P < 0.0001) but was not faster (P = 0.488, Supplementary Figure 2A, B). Similarly, *Anopheles* spent little time moving (Mean = 10.76 %, SD = 10.03) while *Ae. aegypti* was very active (Mean = 79.93 %, SD = 10.03) and *Culex* was moderately active (Mean = 46.71, SD = 16.36, Supplementary Figure 3B). While *Anopheles* spent more than 75% of its time near the surface, *Culex* spent more than 25% in the middle and at the bottom zone of the cuvette and *Ae. aegypti* spent more time at the bottom zone (Supplementary Figure 3A). The difference in activity was maintained when comparing the number of diving event with *Ae. aegypti* and *Culex* diving more than *Anopheles* (both P < 0.0001), but there was no difference between *Ae. aegypti* and *Culex* (P = 0.690, Supplementary Figure 4A). On average, *Ae. aegypti* and *Anopheles* images had

similar surface area (in pixels) (P = 0.438) and were larger than *Culex* (both P < 0.0001, Supplementary Figure 4B).

Finally, movement comparisons between familiarisation and ITI for *Ae. aegypti* showed no difference in average speed (P = 0.834, Supplementary Figure 5A), but a significant difference in maximum speed (P < 0.0001, Supplementary Figure 5B). The comparison of time spent moving show little difference (P = 0.046, Supplementary Figure 5D).

4. Training phase

Learning performance was assessed by comparing individual responses between the 1st and the 10th trials (Figure 3). For the three species, these comparisons rendered significant differences, evincing a decrease in responsiveness (Figure 4). The Performance index was higher at the 1st than at the 10th trial (*Ae. aegypti:* χ^2 = 5.93, df = 1, P = 0.015; *Anopheles:* χ^2 = 9.17, df = 1, P < 0.01; *Culex:* χ^2 = 8.01, df = 1, P < 0.01) (Figure 4A). Vertical distance (mm) was also higher at the 1st than at the 10th trial (*Ae. aegypti:* χ^2 = 13.786, df = 1, P < 0.001; *Anopheles:* χ^2 = 17.957, df = 1, , P < 0.001; *Culex:* χ^2 = 10.472, df = 1, P < 0.01) (Figure 4B).

5. Habituation assessment

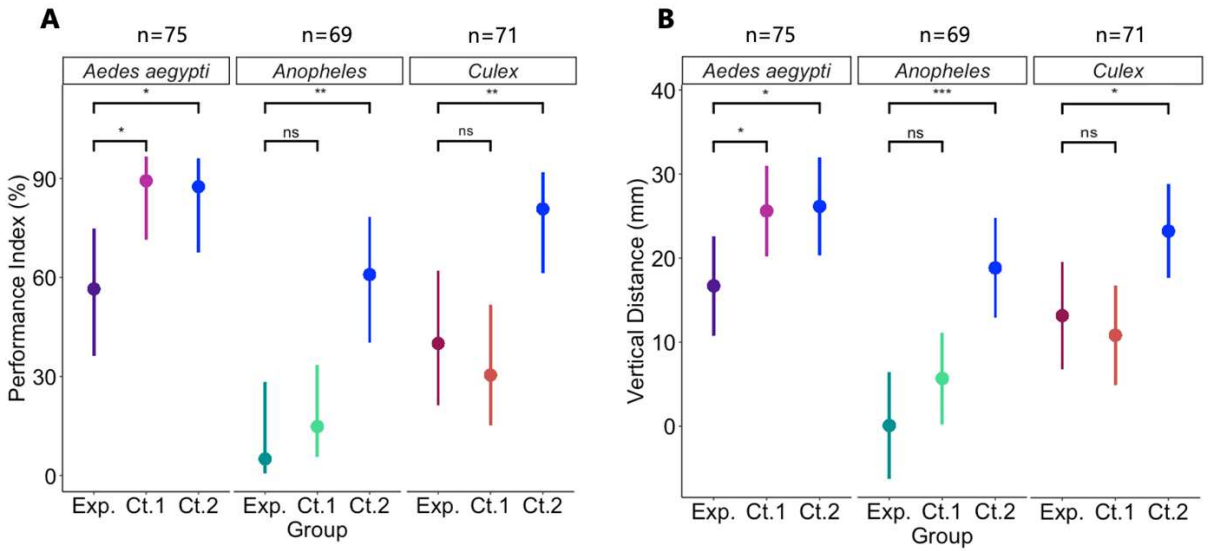


Figure 5: Test phase. A) Performance Index of individuals responding to an aversive stimulus during the test phase (i.e. after the training phase). B) Vertical distance travelled by individuals responding to an aversive stimulus during the test phase. Dark purple, dark green and dark red indicates *experimental* group (Exp.) for each species. Light purple, light green and light red indicates *Control No. 1* (Ct.1) for each

species. Blue indicates *Control No. 2* (Ct.2) for each species. Points indicate mean values and bars indicate +- 95% confidence intervals. NS, not significant; *P<0.05, **P<0.01, ***P<0.001.

For each species, we compared the response at the *test* trial on PI and VD between *Experimental* group, *Control No. 1* and *Control No. 2* (Figures 5, 6). For *Ae. aegypti*, the response level of the *Experimental* group was significantly lower than in *Control No. 1* (PI: 95% CI [0.40, 4.02], P = 0.016; VD: t_{70} = 2.53, P = 0.014) and in *Control No. 2* (PI: 95% CI [0.21, 3.92], P = 0.029; VD: t_{70} = 2.66, P = 0.010, Figure 6). For *Culex* and *Anopheles*, the response of the *Experimental* group was significantly lower than that of *Control No. 2* (*Culex* PI: 95% CI [0.48, 3.32], P = 0.009; VD: t_{64} = 2.07, P = 0.042; *Anopheles* PI: 95% CI [1.17, 6.34], P = 0.004; VD: t_{66} = 4.00, P < 0.001) but not relative to *Control No. 1* (*Culex* PI: 95% CI [-1.75, 0.86], P = 0.507; t_{63} = 0.47, P = 0.643; *Anopheles* PI: 95% CI [-1.23, 3.49], P = 0.347 ; VD: t_{66} = 1.38, P = 0.173), concerning both PI and VD (Figure 5).

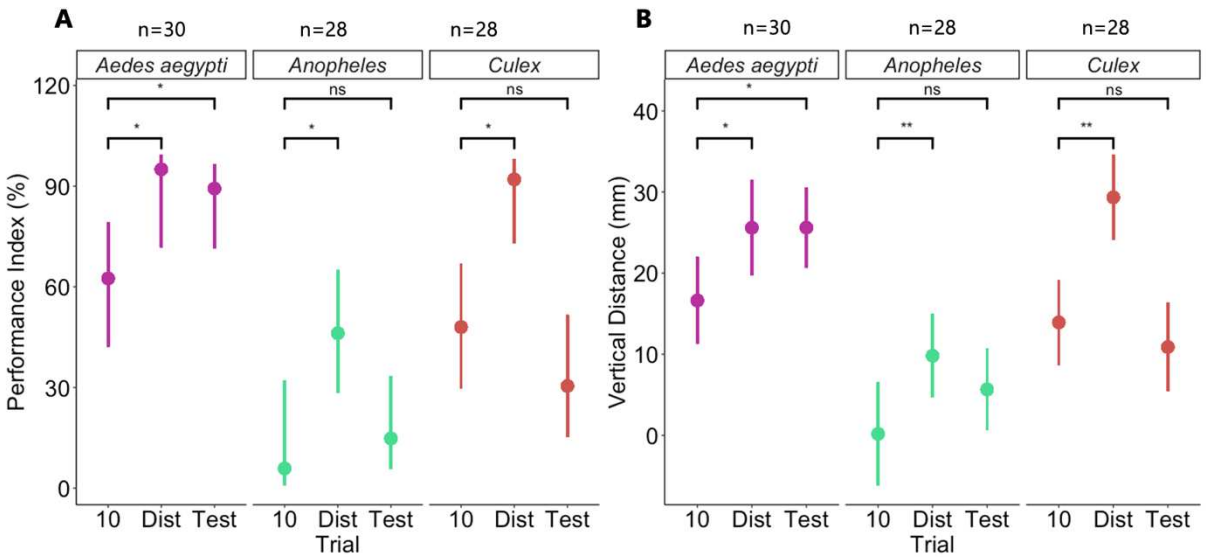


Figure 6: Dishabituation. A) Performance Index of individuals responding to an aversive stimulus in the 10th trial, the disturbance phase and the 12th trial (Test phase). B) Vertical distance travelled by individuals responding to an aversive stimulus in the 10th trial, the disturbance phase and the 12th trial. Dist = disturbance, i.e. the mechanical stimulation between the two trials. Points indicate mean values and bars indicate +- 95% confidence intervals. NS, not significant; *P<0.05, **P<0.01, ***P<0.001.

By comparing individual response between the 10th *training* trial and the *test* phase (i.e, after the disturbance), we looked for evidence of dishabituation to occur. Both, the PI and VD showed contrasted performance for dishabituation across species. *Ae. aegypti* was the only out of the three mosquitoes analysed to show a reversal of the

habituation induced by training (Figure 6). *Culex* and *Anopheles* remained not responsive even after the mechanical disturbance. Yet, all three species evinced an increase in responsiveness when the mechanical disturbance was applied (*Ae. aegypti* PI: 95% CI [0.26, 4.61], P = 0.028; VD: t_{67} = 2.42, P = 0.018; *Culex* PI: 95% CI [0.81, 4.27], P = 0.004; VD: t_{68} = 3.72, P < 0.001; *Anopheles:* PI: 95% CI [0.46, 4.78], P = 0.018; VD: t_{65} = 2.72, P = 0.008; Figure 6). *Ae. aegypti* showed a significant difference between the 10th trial and the *test* phase for both Performance Index and vertical distance (PI: 95% CI [0.15, 3.06], P = 0.030; VD: t_{67} = 2.64, P = 0.010). In contrast, the PI and VD of *Culex* and *Anopheles* were not significantly different between the 10th trial and the *test* phase (*Culex* PI: 95% CI [0.26, 4.61], P = 0.028; VD: t_{68} = -0.75, P = 0.456; *Anopheles:* PI: 95% CI [-1.96, 0.45], P = 0.221; VD: t_{65} = 1.49, P = 0.141; Figure 6).

Discussion

The goal of the present work was to introduce a novel automated system for evaluating the diving response of mosquito larvae, to validate it with insects belonging to different species and having different origins. We showed that the tracking method and the algorithms developed revealed as useful, rendering accurate sets of data and assuring replicability. Automated tracking methods facilitate behavioural quantitative analyses (e.g. Panadeiro et al. 2021). In our work, different behavioural variables could be quantitatively analysed, allowing comparing performances across mosquito species.

We have been able to investigate habituation in mosquito larvae. As expected (Baglan et al. 2017), *Ae. aegypti* larvae were able to habituate to a visual stimulus initially perceived as dangerous, and control groups allowed to distinguish habituation from fatigue and sensory adaptation (Thompson 2009). For *Culex* and *Anopheles,* a significant decrease in the escape response occurred and convergent evidence supported the occurance of habituation in these mosquitoes also. On the one hand, the nature of the stimulus (a passing shadow) and the time elapsed between the last training essay and the test (i.e. several minutes), make sensory adaptation unlikely. On the other hand, the intense response triggered by mechanical disturbance allows excluding motor fatigue.

We calculated two main variables, the Performance Index (PI) and the Vertical Distance (VD) travelled by the larvae. PI was conceived as an easy-to-use binary variable to determine the proportion of individual response to the visual stimulus. This variable is analogous to observations that would have been made by a human experimenter, the major difference was the classification process. By setting a threshold to classify individuals as moving or not on the basis of their relative movement, we avoided classifying brief spontaneous movements or erratic behaviour as positive responses to the visual stimulus. We also ensured that the response interval was constant over the training (i.e. similar interval for each trial). This step was crucial especially for very active species such as *Ae. aegypti* (Jackson 1953; Lutz et al. 2020). A characteristic of the PI is that the threshold was defined in advance, as a minimum intensity of movement for the individual to be considered as responding. In addition, a filter was applied to eliminate 'false zeros' in our zero-inflated model (Zuur et al. 2009), i.e. when individuals could not respond due to their position being at the bottom of the cuvette during the stimulation. Finally, the automated filtering and classification steps provided a robust way to keep constant the selection process over time (i.e. avoiding inter- and intra-observer variability). Thus, quantifying the response of mosquito larvae was based on objective replicable criteria instead of relying on subjective appreciation.

The Vertical Distance (VD) variable was designed to quantify the intensity of the escape response. Upon successive occurrence of the same stimulus, the intensity of a behavioural response may vary or even be completely inhibited (Evans et al. 2019). Here, VD refers to the biological escape response of mosquito larvae, which occurs primarily in the vertical direction, as described by Clements (1999).

Individual displacement was also evaluated in order to quantify spontaneous activity, using the variable Absolute Vertical Distance (AVD), i.e. the total distance travelled during all the ITI periods. Understanding the kinematics of mosquito behaviour using VD or AVD has other advantages. For instance, it allows the interpretation of movement data in a specific context by discriminating between resting period and activity, the direction of displacement, gliding motion, wriggling bouts counts, number of diving events, time spend per area, foraging behaviours, etc (Chandrasegaran et al. 2018; Lutz et al. 2020).

All the three Culicidae studied are part of the neuston (i.e. organisms associated to the water surface, either above or underneath) and, at the same time, they differ in their behaviour. *Ae. aegypti* was the most active during training and the most sensitive to the visual stimulus while *Anopheles* was the least responsive and spontaneously active, and *Culex* was in-between.

Overall, our mosquitoes significantly decreased their response during the training phase. This variation in their responsiveness to a visual stimulus is the result of a trade-off between avoiding predation, maintaining oxygen levels and conserving energy reserves for adult emergence (Awasthi et al. 2012; Baglan et al 2017; Pientrantuono et al. 2021).

All individuals in *Control No. 1* group (i.e., *disturbance*) strongly responded to the mechanical stimulation. While our visual stimulus simulated a flying predator (Tomsic et al. 2009), the mechanical disturbance could illustrate the sudden movement of waves caused by an aquatic predator (e.g. dragonfly larvae, fish, certain mosquito larvae), and could explain the intense response to vibration of the larvae (Clements 1999).

Finally, we found a significant difference in dishabituation in larvae, as has been the case in crabs inhabiting different habitats (see review by Tomsic et al. 2009). Yet, the lack of response at the test phase in *Control No. 1* raises the question on potential differences in learning and memory abilities between species.

In summary, we present here an automated tracking system, which revealed to be reliable, accurate and time-saving, for investigating habituation in mosquito larvae. This learning paradigm proved to be an adequate approach for studying a variety of biological questions related to mosquito cognitive abilities (Baglan et al. 2017, Pientratuono et al. 2021, this paper) as well as the neurological impact of pollutants (Baglan et al. 2018). Other questions which could be addressed using a similar approach range from basic neurobiological mechanisms underlying, for instance memory consolidation and persistence, to ecological problems, as the impact of environmental conditions on cognition.

Acknowledgements

Financial support for this work was granted by Region Centre-Val de Loire, France APR IR 2020 COMPORTATE. M. Dessart is a PhD student at the University of Tours, financed by APR IR 2020 COMPORTATE. COMPORTATE is supported by Réseau MiDi - Milieux et Diversité, Pole DREAM - French Water Team, Zone Atelier de la Loire – LTER France Loire, Réseau ENTOMOCENTRE, Département d'Indre-et-Loire - Direction de l'Attractivité des Territoires (France).

We thank Joël Meunier for material provision and fruitful discussion, David Carrasco for his invaluable advice on statistics, Carole Delavenay for support and the Doctoral School "Santé, Sciences Biologiques, Chimie du Vivant" for guidance and support. The authors express their gratitude to both anonymous reviewers for their constructive criticism and suggestions.

Author contributions

Martin Dessart: Data curation, Formal analysis, Investigation, Methodology, Validation, Software, Visualization, Writing – original draft, Writing – review & editing. **Miguel Piñeirúa:** Data curation, Formal analysis, Methodology, Resources, Software, Software, Validation, Writing – original draft, Writing – review & editing. **Claudio R. Lazzari:** Supervision, Validation, Writing – original draft, Writing – review & editing. **Fernando J. Guerrieri:** Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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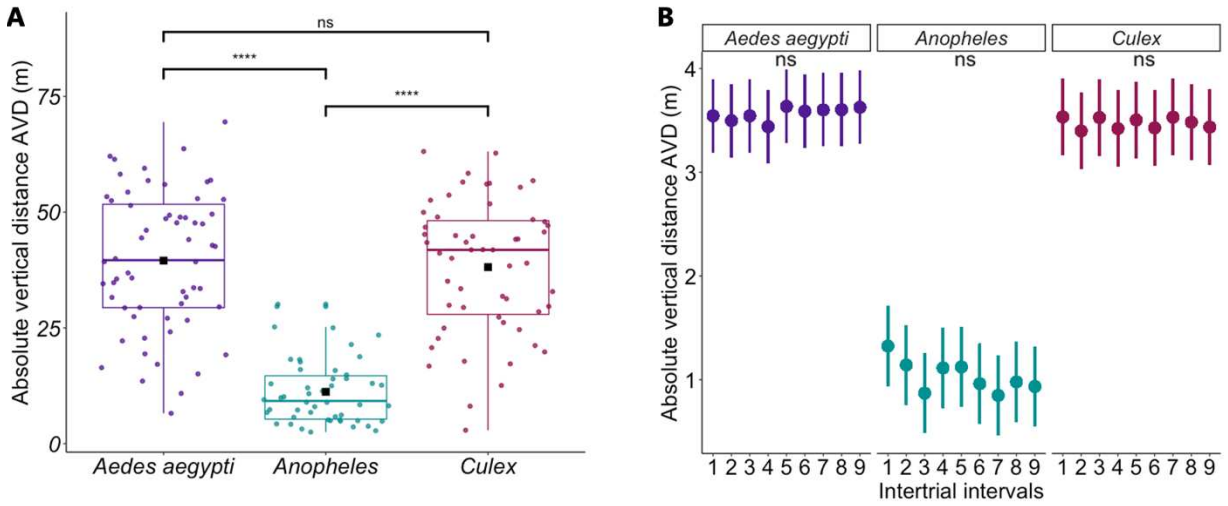
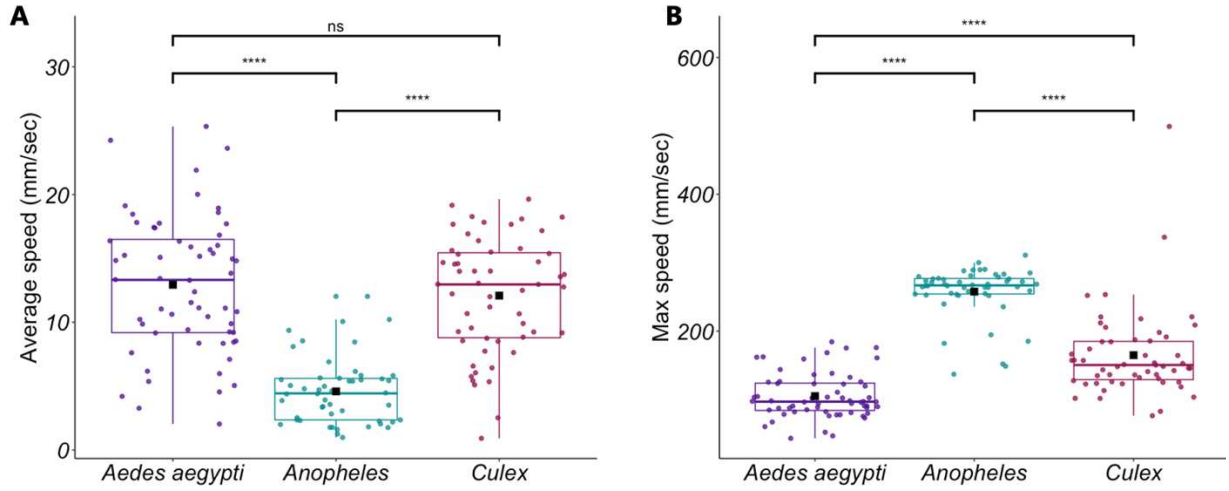
Supplementary material

Replicate	Species	Group	Date	ID number	Detection rate	Vertical length (px)	Comment
1	*Aedes aegypti*	Experimental	Saturday 7 May 2022	10	0.9	390.8	
2	*Aedes aegypti*	Control n°1	Sunday 8 May 2022	10	0.89	391.5	
3	*Aedes aegypti*	Control n°2	Sunday 8 May 2022	10	0.96	393.1	
4	*Aedes aegypti*	Control n°1	Sunday 8 May 2022	10	0.91	386.9	
5	*Aedes aegypti*	Control n°2	Sunday 8 May 2022	10	0.9	389.6	
6	*Aedes aegypti*	Experimental	Monday 9 May 2022	10	0.99	388.4	
7	*Aedes aegypti*	Control n°1	Monday 9 May 2022	10	0.89	391	
8	*Aedes aegypti*	Control n°2	Monday 9 May 2022	10	0.97	393.2	
9	*Aedes aegypti*	Experimental	Thursday 12 May 2022	10	0.94	393.1	
10	*Culex*	Control n°1	Friday 22 July 2022	6	0.93	405.9	ID#5, ID#6, ID#7, ID#8 identified as Culiseta
11	*Culex*	Control n°1	Friday 22 July 2022	5	0.93	401.3	ID#1 to ID#5 did not respond to vibration
12	*Culex*	Control n°1	Friday 22 July 2022	10	0.848	402.3	
13	*Culex*	Experimental	Saturday 23 July 2022	7	0.9	400.3	ID #10 dead, ID#4 and ID#9 did not respond to vibration
14	*Culex*	Control n°1	Thursday 18 August 2022	8	0.95	404.2	ID#1 and ID#2 did not respond to vibration
15	*Culex*	Experimental	Thursday 18 August 2022	9	0.95	407.9	ID#8 did not respond to vibration
16	*Culex*	Experimental	Thursday 18 August 2022	10	0.93	407.2	
17	*Culex*	Control n°2	Thursday 18 August 2022	9	0.92	404.6	ID#2 did not respond to vibration
18	*Culex*	Control n°2	Thursday 18 August 2022	9	0.95	393.4	ID #10 dead
19	*Culex*	Control n°2	Thursday 18 August 2022	9	0.87	392.1	ID #3 dead
20	*Anopheles*	Control n°1	Tuesday 2 August 2022	10	0.99	409.2	
21	*Anopheles*	Experimental	Tuesday 2 August 2022	8	*	412.9	ID #3 dead, ID #10 not visible
22	*Anopheles*	Control n°1	Tuesday 2 August 2022	9	*	411.3	ID#8 did not respond to vibration
23	*Anopheles*	Experimental	Tuesday 2 August 2022	7	*	412.8	ID#2, ID#3, not visible, ID#7 did not respond to vibration
24	*Anopheles*	Control n°1	Tuesday 2 August 2022	9	*	417.5	ID #5 dead
25	*Anopheles*	Control n°2	Wednesday 3 August 2022	8	*	411.6	
26	*Anopheles*	Control n°2	Thursday 3 August 2023	10	0.87	412	
27	*Anopheles*	Experimental	Wednesday 17 August 2022	7	*	408.4	ID#2, ID#4, ID#6 did not respond to vibration
28	*Anopheles*	Control n°2	Wednesday 17 August 2022	6	0.97	405.9	ID#2, ID#5, ID#9 not visible, ID #10 dead
			Total	246	92.54%	401.4	*Individuals not moving --> detection rate not calculable

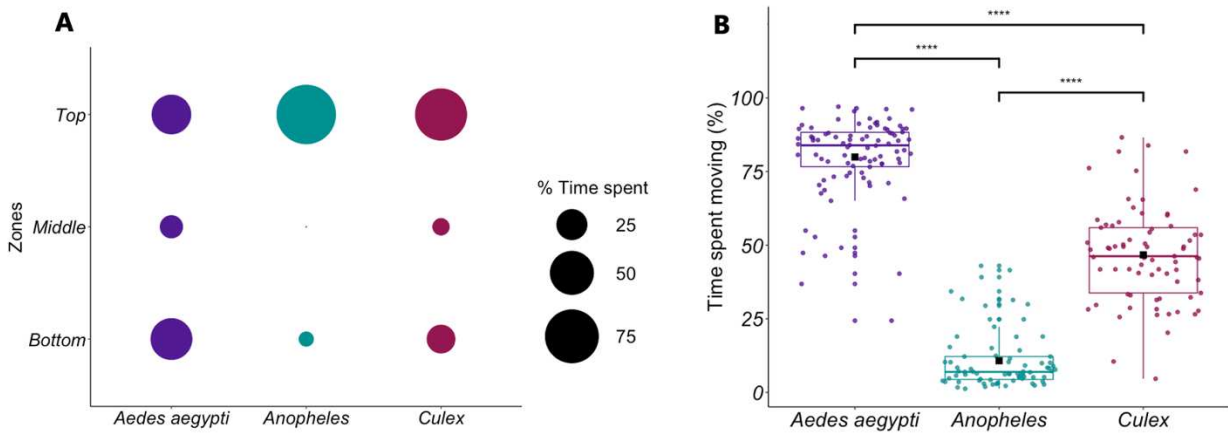
Supplementary Table 1: Details for each experiment performed. ID number corresponds to the active number of individuals kept for the analysis. Detection rate was calculated as the ratio between the maximum frame number and the actual frame number identified by the tracking software. Vertical length was calculated as the difference between the maximum and the minimum individual position measured on each video by the tracking software.



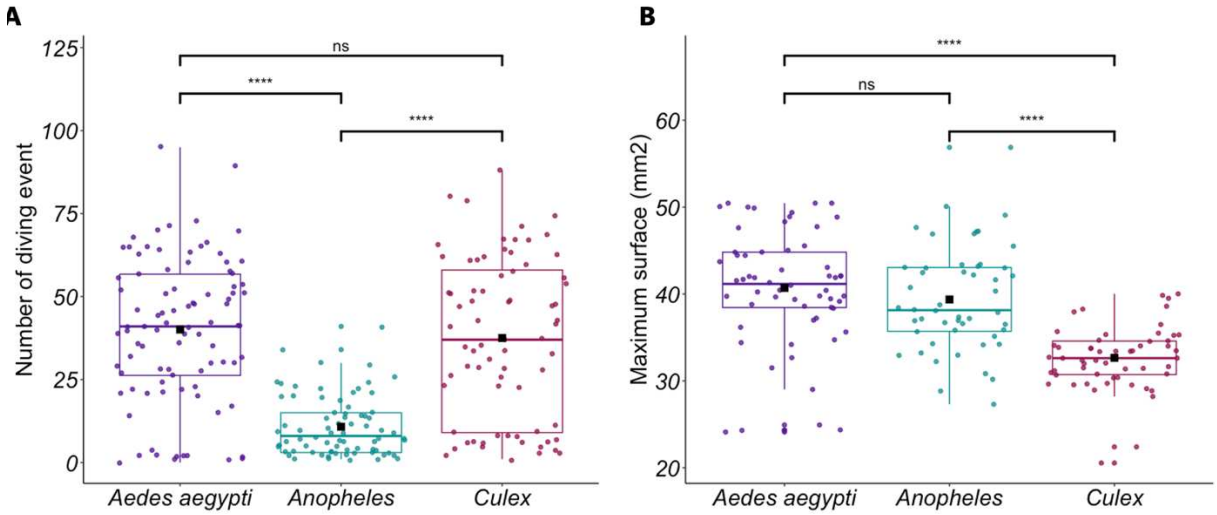
Supplementary Figure 1: Total distance travelled during the training period. A) Total AVD for each individual during the training period. B) AVD travelled by each individual for each inter-trial interval. Boxplots show the median values (line) and interquartile ranges. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one species. NS, not significant; ****P<0.0001.



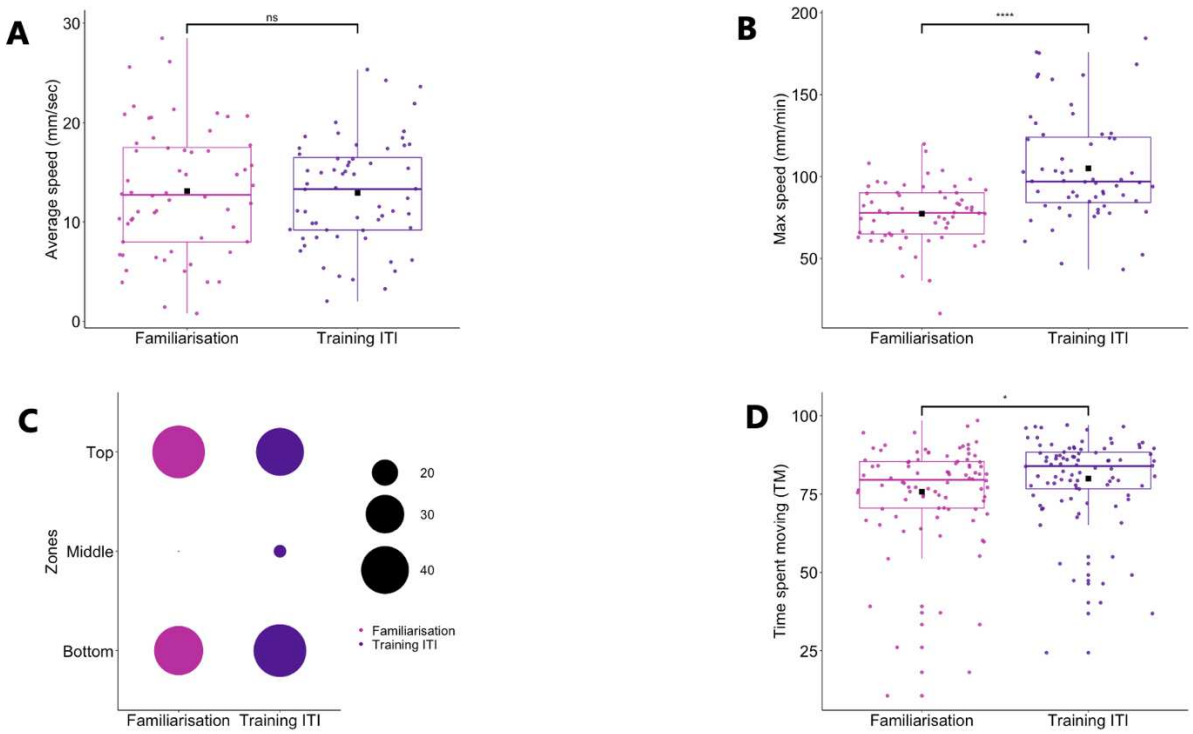
Supplementary Figure 2: Average speed and maximum speed. A) Average speed for each individual during the training period. B) Maximum speed for each individual during the training period. Boxplots show the median values (line) and interquartile ranges. Points indicate mean values. Dark square indicates mean value for one species. NS, not significant; ****P<0.0001.



Supplementary Figure 3: Time spend per zone and moving. A) Average time spend per zone (TZ) for each individual during the training period. B) Average time spend moving TM for each individual during the training period. Boxplots show the median values (line) and interquartile ranges. Points indicate mean values. Dark square indicates mean value for one species. NS, not significant; ****P<0.0001.



Supplementary Figure 4: Number of diving event and average size. A) Number of diving event for each individual during the training period. B) Maximum surface detected by the tracking software for each individual. Boxplots show the median values (line) and interquartile ranges. Points indicate mean values. Squares indicate group mean value. NS, not significant; ****P<0.0001.



Supplementary Figure 5: Comparison between familiarisation and training activity for *Aedes aegypti.* A) Average speed for each individual. B) Maximum speed for each individual. C) Average time spend per zone (TZ) for each individual. D) Average time spend moving TM for each individual. Boxplots show the median values (line) and interquartile ranges. Points indicate mean values. Squares indicate group mean value. NS, not significant; *P<0.05, ****P<0.0001.

Chapitre 3 : Avoir la mémoire courte : évaluation de la persistance de la mémoire chez les larves de moustique

One question that needs ultimately answered is as follows: How do we go about measuring the knowledge stored in memory? We simply do not know (yet?)

Randolf **Menzel** (2022) In Search for the Retrievable Memory Trace in an Insect Brain

Chez les animaux, la **mémoire** est primordiale d'un point de vue adaptatif car elle facilite, accélère et spécifie la prise de décision sur la base d'une expérience vécue. Elle permet d'éviter des situations dangereuses, de se souvenir de lieux importants et d'économiser de l'énergie en ne réagissant pas à des stimuli inutiles. Cependant, la formation et le maintien de la mémoire ont un **coût métabolique important**, ce qui questionne sur les mécanismes qui sous-tendent les différents types de mémoire et leur valeur adaptative. Dans ce chapitre, nous avons exploré **la persistance de la mémoire** des larves de moustique ***Aedes aegypti*,** après **habiuation** à un stimulus visuel simulant un prédateur en approche. En particulier, nous avons étudié le rôle de **l'intervalle entre essais** dans la formation et la rétention de la mémoire. Contrairement à d'autres invertébrés aquatiques, les larves de moustique n'ont fait preuve que de mémoire à court terme, même avec des intervalles entre essais longs (c'est-à-dire de 10 minutes). Nos résultats sont discutés en relation avec les **contraintes écologiques** des larves de moustique.

Ce chapitre a fait l'objet d'une publication dans : **Journal of Insect Physiology** et figure en annexe de ce manuscrit de thèse.

Dessart M, Lazzari C, Guerrieri F (2024) Habituation leads to short but not long term memory formation in mosquito larvae. Journal of Insect Physiology.155:104650. https://doi.org/10.1016/j.jinsphys.2024.104650

Habituation leads to short but not long term memory formation in mosquito larvae

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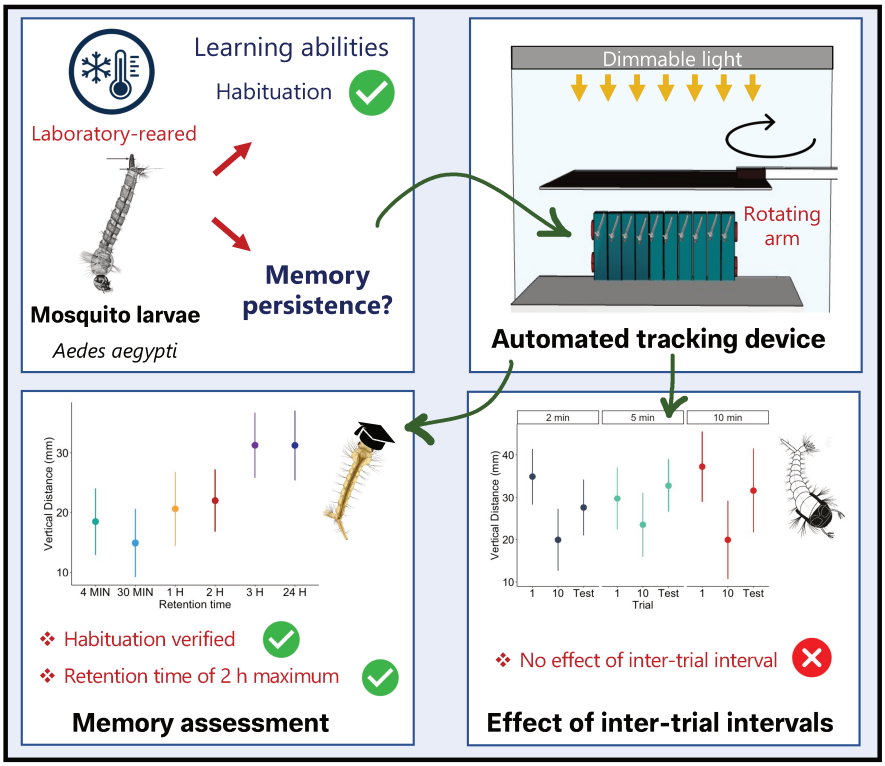
Keywords: habituation, non-associative learning, *Aedes aegypti,* short-term memory, inter-trial interval

Highlights

1. Habituation to a visual stimulus was used to study memory in mosquito larvae
2. Different inter-trial intervals were applied to establish memory retention
3. No retention was observed beyond two hours after training
4. No long-term memory could be induced

Graphical abstract



Abstract

In animals, memory allows to remember important locations and conserve energy by not responding to irrelevant stimuli. However, memory formation and maintenance are metabolically costly, making it worthwhile to understand the mechanisms underlying different types of memory and their adaptive value. In this study, we investigated the memory persistence of *Aedes aegypti* mosquito larvae, after habituation to a visual stimulus. We used an automated tracking system for quantifying the response of mosquito larvae to the passage of a shadow, simulating an approaching predator. First, we compared different retention times, from 4 min to 24 h, and found that mosquito larvae only exhibited memory capabilities less than 3 hours after training. Secondly, we investigated the role of inter-trial intervals in memory formation. In contrast to other aquatic invertebrates, mosquito larvae showed no long-term memory even at long inter-trial intervals (i.e., 5 min and 10 min). Our results are discussed in relation to the ecological constraints.

Introduction

To retain information, or memory, is a crucially adaptive cognitive ability in animals (Menzel 1999). The adaptive value of memory is related to the ability to make quick and accurate decisions when faced with a situation similar to one previously experienced (Menzel and Benjamin 2013). Memory allows animals to avoid harmful situations, to remember important locations or specific information, and to avoid energy loss by not responding to irrelevant stimuli; in other words, memory contributes to overall fitness (Couto et al. 2023). At the same time, memory formation and maintenance have different costs (Niven and Laughlin 2008). As the brain is metabolically expensive, the resources allocated to encode, consolidate, and access information generate important expenditures (Kandel 2001). Different types of memory coexist, defined by their duration and the physiological processes involved in their development. They end up being adaptative or not depending on the context. For instance, in stable environments, where the probability of encountering a certain situation again is high, it may be adaptative to invest in long-term memory. In a rapidly changing environment, however, it may be better to prioritise short-term memory (Pull et al. 2022).

The properties and the physiological mechanisms underlying the different types of memory have been studied in many invertebrate models, notably in the fruit fly *Drosophila melanogaster* (Tully 1994) and the honey bee *Apis mellifera* (Menzel 2001a). In addition, habituation to visual stimuli and memory has been well characterised in the mudflat crab *Neohelice granulata* (Tomsic and Silva 2023). These experiments provided insights about the ecological relevance of memory duration according to the habitat. In a study by Tomsic et al. (1993), the authors compared the habituation of two related semi-terrestrial crabs that occupy different habitats, *Neohelice granulata* and *Pachygrapsus marmoratus*. By analysing the influence of diverse parameters on visual habituation performances (e.g., individual size, number of trials), the authors showed that habituation is species-dependent and that contextual cues are memorised differently. Tomsic et al. (1993) concluded that ecology played a major role in the origin of these differences. Indeed, *Neohelice granulata* crabs live in self-dug burrows, closed to the mud substrate and surrounded by conspecifics and halophyte vegetation. On the other

hand, *Pachygrapsus marmoratus* live on rocky outcrops, close to the sea and without vegetation. So, a shadow passing over *Neohelice* crabs would induces stronger and longer habituation because it represents an ambiguous signal (e.g., grass undulation), whereas for *Pachygrapsus* crabs, the probability of being an actual flying predator would be higher in their environment which is poor in objects passing overhead (Tomsic et al. 1993), resulting in a weak habituation response in the latter.

A key parameter for habituation and the mesic mark it can generate, is the inter-trial interval (Giurfa et al. 2009). Short inter-trial intervals (e.g., from few seconds to few minutes) are more likely to reinforce short-term memory, which relies on neural facilitation (i.e., increase in synaptic strength) and reversible changes (Hemmi and Tomsic 2012), but not long retention. In contrast, long inter-trial intervals will lead to the formation of long-term memory, which depends on the activation of specific genes leading to new protein synthesis and structural changes in neural circuits (Tomsic et al. 1996; reviewed in Margulies et al. 2005 in Drosophila). In between, intermediate inter-trial intervals produce intermediate memory, which involves synaptic consolidation through the activation of specific kinases (e.g., cAMP-dependent protein kinase PKA) and early gene expression (Tomsic and Romano 2013). While the duration of inter-trial intervals has been empirically tested, these types of memory have also been described in several taxa (Tully 1994; Izquierdo et al. 1998; Menzel 2001b).

In this work, we investigated the ability to develop memory after learning in an aquatic insect, the mosquito larva (*Aedes aegypti*). Mosquito larvae spend most of their time hanging from the water surface. When a stimulus is perceived as a potential danger, larvae dive (Clements 1999). If the stimulus turns out to be innocuous upon repeated occurrences, larvae no longer respond to further stimulation due to habituation, a form of non-associative learning, potentially forming a mnesic trace (Baglan et al. 2017; Dessart et al. 2023).

Although much attention has been paid to cognition in adult mosquitoes, this is the first study to investigate the memory of mosquito larvae. In freshwater ecosystems, mosquito larvae are part of the neuston (i.e., organisms living at the water surface). They are therefore surrounded by unpredictable aquatic and aerial predators such as dragonfly larvae or water striders (for review see: Vinogradov et al. 2022). In this type of environment, a shadow repeatedly casting over the water surface in a short period of time

is likely to be projected by the same object, whereas a shadow projected over the water hours later could be produced by a different moving body. In this situation, we could expect that mosquito larvae stop to respond to the repetition of an aversive stimulation in the short term, while resetting their responsiveness in the long term, i.e., not to remember, would be a more adaptive strategy.

In addition to very-well studied aquatic invertebrates such as the sea hare *Aplysia californica* (Glanzman 2009) or the crab *Neohelice granulata* (Tomsic et al. 2017) which exhibit remarkable forms of long-term memory, other freshwater organisms also showed consistent long-term memories, as for example crayfish *Procambarus cubensis* up to 24 hours (Abramson et al. 2005), great pond snails *Lymnaea stagnalis* up to 3 days (Lukowiak et al. 2003), and water fleas *Daphnia sp*. up to 6 days (Ringelberg and Gool 1995). Since long-term memory has been demonstrated in several aquatic species, the possibility of long-term memory in mosquito larvae cannot be ruled out without experimental evidence.

On the one hand, the highly unpredictable environment could prioritise the formation of a short-term memory in mosquito larvae. On the other hand, other organisms from similar environments show robust long-term memory. To distinguish between these two hypotheses, we conducted a series of experiments with *A. aegypti* mosquito larvae to investigate (1) how long mosquito larvae could retain information after habituation, and (2) whether the duration of inter-trial intervals would play any role in memory formation.

Material and methods

1. Animals

A. aegypti eggs (Bora strain) were provided by the INFRAVEC2 project of MIVEGEC-IRD (Montpellier, France). The eggs were reared in a climate-controlled room at 25°C ± 2°C and under 12h:12h light:dark illumination regime (lights on at 8:00). The larvae were maintained in small plastic containers filled with dechlorinated tap water and fed *ad libitum* with shrimp food (JBL Novo Prawn, Neuhofen, Germany). Fourth-instar larvae were used in all the experiments to maximise the chances of encountering robust

cognitive abilities. In addition, larger larvae allow a more precise tracking. All animals were reared and manipulated according to ethics regulations applied in the European Union.

2. Experimental apparatus

The experimental apparatus consisted of two light sources, a camera, and a servo mechanism, which controlled the projection of the shadow of a square cardboard (shadow) above twelve 1.5 ml spectrophotometer plastic cuvettes (Z187992-1PAK, Sigma-Aldrich, Germany) where the larvae had been individually placed. One light source consisted of two LED panels (30 cm x 30 cm), located above the cuvettes (Figure 1A). The second light source was an infrared backlight (880 nm) placed behind the cuvettes. In front of the cuvettes, a camera (acA 1300 – 60gc, Basler, Germany) equipped with a high-pass infrared filter (RG 850 Filter - 40.5 mm, Heliopan, US) recorded the experiments (for more details, see Dessart et al., 2023). The projected shadow induced naive larvae to dive vertically, escaping from potential danger. After repeated presentations of the shadow, the escape response decreased due to habituation, a form of non-associative learning (Dessart et al. 2023).

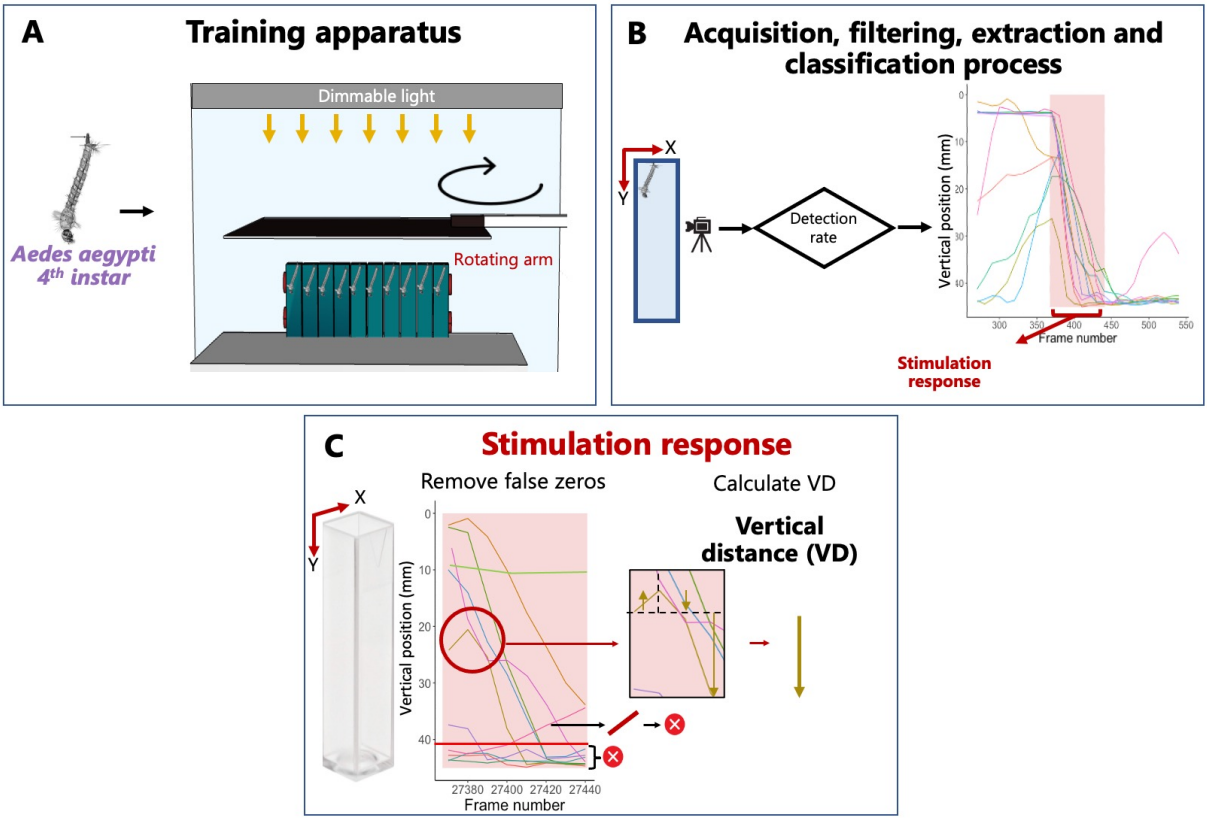


Figure 1: Schematic of the experimental protocol. A) *Aedes aegypti* larvae were reared in the laboratory and trained on the apparatus at the fourth larval. B) Experiments were video-taped and individual trajectories were extracted. C) We analysed the behavioural response during the aversive stimulus, using the metric vertical distance (VD). This variable was quantitative and calculated as the relative sum of the distance travelled vertically towards the bottom of the cuvette. In addition, two filters were applied to exclude individuals that were at the bottom of the cuvette during the first few frames of the stimulus and individuals that moved upwards during the 3-second stimulus period.

3. Experimental procedure

The experimental procedure included a training phase and a test phase. During the training phase, individuals were presented with a shadow 10 consecutive times (trials), separated by a specific inter-trial interval (ITI). The stimulus was a black cardboard square (16 cm side) attached to a wooden stick and fixed to a servomotor controlled by an Arduino Uno board. During a trial, the stick turned 100◦ and returned back to the resting position, during 3 s. After the 10th shadow presentation, the larvae rested for a specific period (retention time) before a final presentation of the shadow (test phase).

Replicate	Group	Retention	ITI	ID number	Detection rate	Vertical length (px)	Comment
7	1	4 min	2	10	0.82	399.4	
10	1	4 min	2	10	0.83	403.8	
12	1	4 min	2	10	0.95	401.6	
8	2	30 min	2	10	0.93	403.3	
9	2	30 min	2	10	0.92	400.5	
13	2	30 min	2	10	0.96	400.7	
11	3	1h	2	10	0.97	400.6	
14	3	1h	2	10	0.89	404.3	
16	3	1h	2	9	0.93	401.1	ID#10 not tracked
15	4	2h	2	9	0.98	406.6	ID#6 never moved at all
17	4	2h	2	10	0.97	400.8	
18	4	2h	2	10	0.92	405.6	
19	5	3h	2	9	0.97	400.8	ID#7 transformed in pupae
20	5	3h	2	10	0.9	399.7	
21	5	3h	2	10	0.98	404	
2	6	24h	2	10	0.95	401.9	
4	6	24h	2	10	0.99	403.4	
3	7	24h	5	9	0.85	403.6	ID#8 not tracked
5	7	24h	5	10	0.90	400.8	
1	8	24h	10	10	0.98	400	
6	8	24h	10	10	0.92	401.8	

Table T1: Details of the two experiments conducted. Each replicate represents 10 individuals (or less where indicated) trained during one session. The retention column refers to the time between training and test. ITI = inter-trial interval used during the session. ID number corresponds to the number of individuals for each replicate. Detection rate was calculated as the ratio between the maximum number of frames and the actual number of frames identified by the tracking software. Vertical length was calculated as the difference between the maximum and the minimum individual position measured by the tracking software on each video.

Two experiments were set up to evaluate the duration of memory and possible effects of ITI on the duration of memory. First, 6 treatments of 20 - 30 individuals were established, each trained with 2-min ITI and tested at different times after the training, ranging from 4 min to 24 hours (i.e., Treatment 1 = 4 min, Treatment 2 = 30 min, Treatment 3 = 1h, Treatment 4 = 2h, Treatment 5 = 3h, Treatment 6 = 24h, Table 1). These intervals were chosen to investigate the memory persistence of mosquito larvae. The order of the treatments was pseudorandomised. Then, a new set of larvae were trained with 2 min ITI, 5 min ITI or 10 min ITI, and retention tested at 24-hour (i.e., Treatment 6 = 2 min ITI, Treatment 7 = 5 min ITI, Treatment 8 = 10 min ITI, Table 1). The order of the treatments was also pseudorandomised, and six replicates of 10 individuals were trained per day, resulting in 6 consecutive days of training (Table 1).

Training and testing took place in the afternoon, from 12h to 19h. Individuals were removed from the database if they transformed into pupae during the training or retention period (n = 1), if they remained motionless throughout the entire training period

(n = 1) or if the tracking failed to distinguish the individual (n = 2) (Table 1). A total of 205 individuals from 21 replicates were kept for the analysis.

4. Data analysis

Each replicate was video recorded, and the individual trajectory was extracted using a tracking algorithm previously used by Dessart et al. (2023) (Figure 1B). We first applied a detection rate by comparing the number of frames successfully identified by the tracking algorithm with the theoretical maximum number of frames. All videos were analysed with a minimum detection rate of 82.5% (Table 1). Two analyses were then performed on individual trajectory.

To assess learning and memory abilities, we considered the stimulus response corresponding to the behavioural response of individuals during the 3-seconds trial period of the shadow passage (Figure 1C). Using these data, we first excluded individuals that were at the bottom of the cuvette at the start of a trial (below 9/10th of the cuvette length, 26.1% of trials removed, Table 2). We then calculated the variable Vertical Distance (VD) as the vertical downward distance travelled by each individual during the 3 seconds stimulus onset (Dessart et al. 2023). Using VD, we excluded individuals that travelled to the top of the cuvette during a trial (i.e., that travelled more than 10 mm upwards, 1.8% of trials removed, Table 2). A total number of 205 individuals and 1622 trials were retained for the analysis (Table 2).

			2 MIN ITI				*5 MIN ITI*	*10 MIN ITI*	All
	4 MIN	**30 MIN**	**1 H**	**2 H**	**3 H**	**24 H**	**24 H**	**24 H**	**Total**
Individuals trained	30	30	29	29	28	20	19	20	205
Trials per individuals	320	330	319	319	308	220	199	220	2235
Trials filtered by position	236	253	212	256	236	159	147	152	1651
% Trials removed	26.3%	23.3%	33.5%	19.7%	23.4%	27.7%	26.1%	30.9%	**26.1%**
Trials filtered by going up	230	250	205	255	235	158	140	149	1622
% Trials removed	2.5%	1.2%	3.3%	0.4%	0.4%	0.6%	4.8%	2.0%	1.8%
Total % Trials removed	28.1%	24.2%	35.7%	20.1%	23.7%	28.2%	29.6%	32.3%	**27.4%**

Table T2: Summary of the filtering steps. Between 19 and 30 individuals were trained for each species. If the individual's position was near the bottom at the beginning of a trial, the response to that trial was removed, accounting for a total of 26.1% of removed trials. Individuals that moved upwards during a trial were also removed for that particular trial, accounting for 1.8% of trials.

Statistical analyses

1. Data sharing

All the results were analysed using R version 4.1.1 (2021-08- 10) (https://cran.r-project.org/). The data and the R programs used in this study are available at: https://github.com/martindessart/Brain_Like_A_Sieve.

2. Data filtering

For all treatments, we verified that number of trials deleted by the criterion did not depend on the trial number (similar as Dessart et al. 2023). Briefly, we applied a Chi-square goodness of fit test to verify that the larval position did not change (i.e., increase or decrease) across trials.

3. Learning and memory performance

We first modelled learning performance using Generalised Additive Model (GAM) to provide a visual estimate of the training period. We defined models of increasing complexity and different smoothing functions and compared them using the GCV-UBRE in the *mgcv* package (Wood 2017). We then evaluated the learning performance of each treatment by comparing the response in the 1st trial to the response in the 10th trial and in the Test phase. For each treatment, we used a linear mixed effects model, choosing VD as the response variable, trial as fixed factor and individual identity as random factor. We checked the homogeneity of the distribution of variances and residuals using the *DHARMa* package (Hartig 2022). We evaluated the pairwise comparisons using the *emmeans* package with Tukey correction for 3 estimates (Lenth 2021).

4. Test comparison

To compare the duration of memory across treatments, we compared the response in the Test phase by using a linear mixed effects model with VD as the response variable, the retention time as fixed factor and individual identity as random factor. We followed the same procedure as before to assess pairwise comparisons.

5. Learning efficiency

We also estimated how quickly larvae would significantly decrease their response as a function of the ITI, to estimate how many trials would be sufficient to induce a significant decrease in response. To answer, we compared the response at the 1st and the 2nd trial, using a linear mixed effects model as described above.

Results

1. Data filtering

To analyse the global position of the larvae over time, we compared the proportion of deleted trials by the two criteria between trials (similarly as Dessart et al. 2023). For each treatment, the deleted trials were not specific to a trial number: Treatment 1: χ^2 = 7.53, df = 10, P = 0.674; Treatment 2: χ^2 = 3.6, df = 10, P = 0.964; Treatment 3: χ^2 = 3.91, df = 10, P = 0.951; Treatment 4: χ^2 = 6.81, df = 10, P = 0.743; Treatment 5: χ^2 = 6.41, df = 10, P = 0.780; Treatment 6: χ^2 = 9.68, df = 10, P = 0.469; Treatment 7: χ^2 = 15.02, df = 10, P = 0.131; Treatment 8: χ^2 = 6.93, df = 10, P = 0.732.

2. Learning performance

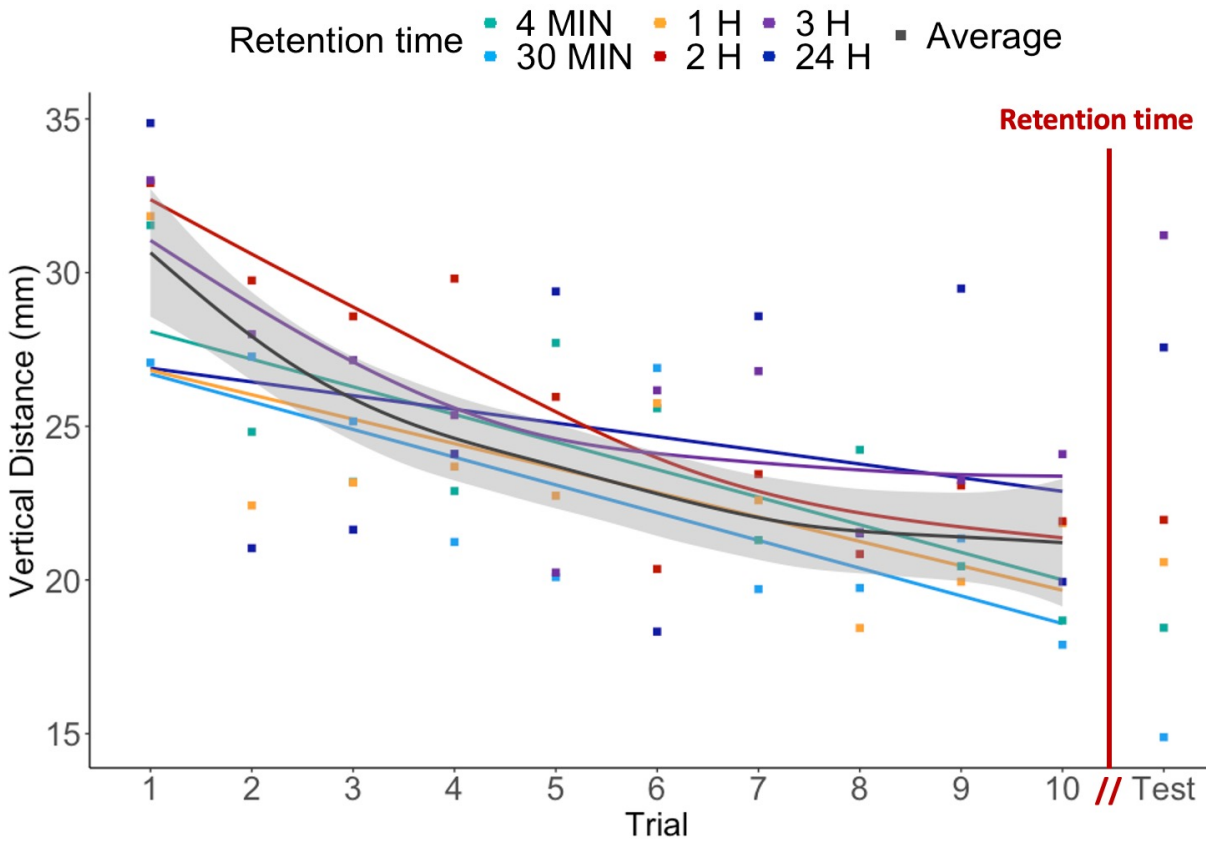


Figure 2: Habituation curves for larvae trained with 2 min inter-trial interval and with several retention times. 4 min (green), 30 min (cyan), 1 hour (yellow), 2 hours (red), 3 hours (purple), 24 hours (dark blue) retention time. Average in black. Vertical distance (in millimetres) corresponds to the distance covered by an individual during the stimulus period, from the 1st to the test phase. Red vertical line corresponds to the retention time. Smoothing lines indicate the best-fitting GAM model. Grey shading indicates 95% confidence interval for the average response. Points indicate mean values.

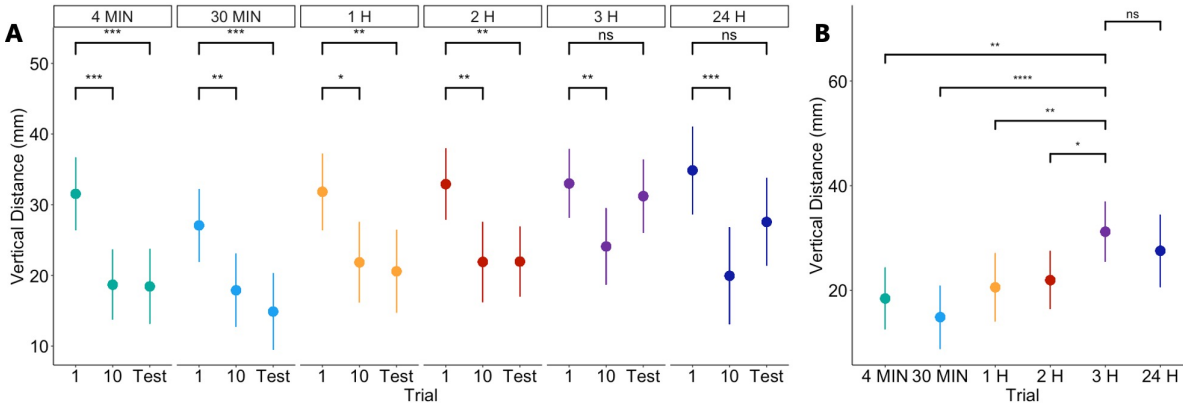


Figure 3: Mosquito larvae retain visual information up to 2 hours after habituation. A)
For each retention time, vertical distance in millimetres travelled by individuals responding to an aversive stimulus during the 1st, the 10th and the test trial. B) Comparison of the vertical distance travelled by individuals during the test trial. 4 min (green), 30 min (cyan), 1 hour (yellow), 2 hours (red), 3 hours (purple), 24 hours (dark blue). Points indicate mean values and bars indicate +- 95% confidence intervals. NS, not significant; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

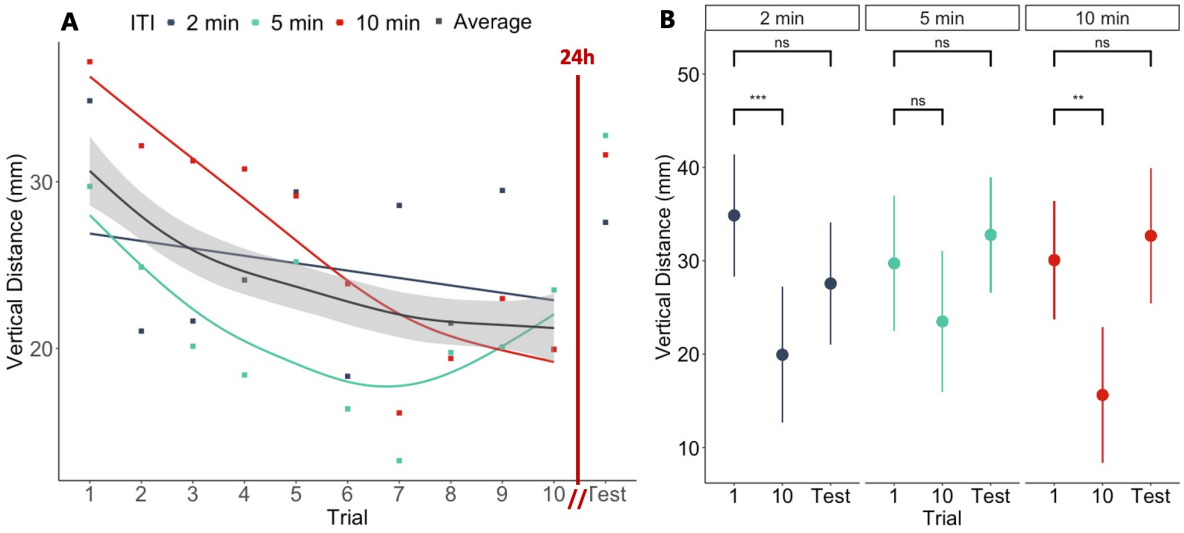


Figure 4: Learning and memory performance of individuals is independent of the inter-trial interval. A) Habituation curves for individual training with 2 min (dark grey), 5 min (green), 10 min (dark red) inter-trial intervals. Average in black. Vertical distance (in millimetres) corresponds to the distance covered by an individual during the stimulus period, from the 1st to the test phase. Red vertical line corresponds to the retention time of 24 hours. Smoothing lines indicate the best-fitting GAM model. Grey shading indicates 95% confidence interval for the average response. B) For each inter-trial interval, the vertical distance in millimetres travelled by individuals responding to an aversive stimulus during the 1st, the 10th and the Test trial. Points indicate mean values and bars indicate +- 95% confidence intervals. NS, not significant; **P<0.01, ***P<0.001.

For all treatments, the behavioural response of the larvae decreased with successive stimulations (Figure 2, Figure 4A). To model this behavioural response, the P-spline from GAM models was the best smoothing function, similar to as previous work (Dessart et al. 2023). For all treatments except Treatment 7, the Vertical distance VD was higher in the 1st than in the 10th trial: Treatment 1: t-ratio = 3.943, df = 45, P < 0.001; Treatment 2: t-ratio = 2.590, df = 46, P = 0.03; Treatment 3: t-ratio = 2.626, df = 37, P = 0.03; Treatment 4: t-ratio = 2.704, df = 49, P = 0.03; Treatment 5: t-ratio = 2.540, df = 46, P = 0.04; Treatment 6: t-ratio = 2.957, df = 30, P = 0.02; Treatment 8: t-ratio = 3.315, df = 28, P < 0.01 (Figure 3A, Figure 4B). For Treatment 7 (i.e., ITI = 5 min and retention time = 24 hours), although the response decreased after the 1st trial, the difference between the 1st and the 10th trial was not significant: t-ratio = 1.088, df = 32, P = 0.53 (Figure 4B). This difference may be due to the small number of trials retained for this comparison (1st trial: n=13; 10th trial: n=12).

3. Effect of the retention time on memory performance

To investigate how long the information from the training would be stored in the mosquito larval brain, we compared the response during the Test phase for different retention times. First, we compared the response at the 1st trial to the Test phase (Figure 3A). For retention times up to 2 hours, the response in the Test phase was lower than in the 1st trial: Treatment 1: t-ratio = 3.823, df = 46, P < 0.01; Treatment 2: t-ratio = 3.354, df = 46, P < 0.01; Treatment 3: t-ratio = 2.529, df = 37, P = 0.04; Treatment 4: t-ratio = 2.906, df = 45, P = 0.02; Treatment 5: t-ratio = 0.520 , df = 46, P = 0.86; Treatment 6: t-ratio = 1.533, df = 29, P = 0.30 (Figure 3A). We also compared the response at the Test phase between the highest response (Treatment 5 = 3h) with other Treatments (Figure 3B). The response at the Treatment 5 was higher than Treatment 1: t-ratio = 3.097, df = 100, P < 0.01, Treatment 2: t-ratio = 3.874, df = 101, P < 0.001, Treatment 3: t-ratio = 2.366, df = 103, P = 0.02 and Treatment 4: t-ratio = 2.319, df = 98, P = 0.02. The response at the Treatment 6 was no different from Treatment 5: t-ratio = 0.704, df = 105, P = 0.48, Figure 3B).

4. Effect of intertrial interval on long-term memory

As the larvae showed no retention after 3 hours, we investigated whether 2 min ITI was sufficient to induce a long-term memory. We trained larvae using the same procedure, but with an increased ITI of 5 min and 10 min. We compared the response in the Test phase after 24 hours of retention time for 3 ITI: 2 min, 5 min and 10 min. The difference between the 1st trial and the Test phase was not significant for any of the three Treatments: 2 min ITI: t-ratio = 1.533, df = 29, P = 0.29; 5 min ITI: t-ratio = -0.596, df = 28, P = 0.82; 10 min ITI: t-ratio = -0.550, df = 25, P = 0.85 (Figure 4B).

5. Effect of intertrial interval on learning efficiency

To characterise the speed of learning, we compared the response between the 1st and the 2nd trials for the 3 ITIs. The difference between the 1st and the 2nd trials was only significant for the 2-min ITI: t-ratio = 2.955, df = 15, P < 0.01; 5 min ITI: t-ratio = 0.836, df = 13, P = 0.42; 10 min ITI: t-ratio = -0.069, df = 16, P = 0.94 (Figure S1).

Discussion

In this work, we took advantage of an automated system to investigate memory persistence in the 4th instar of *A. aegypti* mosquito larvae. Two distinct questions were addressed: how long mosquito larvae could retain information following a habituation learning paradigm, and whether habituation training with longer inter-trial intervals would induce long-term memory in mosquito larvae or not. In the first part of this study, we found that following a habituation protocol, mosquito larvae could retain the information for up to 2 hours after 10 trials with 2 min ITI. Yet, no memory was found after 3 hours and 24 hours of retention. In the second part of this work, we focused on the effect of ITI on the memory persistence at 24 hours after training. Equally, we found no long-term memory at 2 min ITI, 5 min ITI and 10 min ITI.

These results support the hypothesis that the ecological context of mosquito larvae would favour short-term memory (Juliano 2009). First, mosquito larvae live in a wide range of unpredictable environments, where visually hunting predators could be abundant, the structure of the habitat complex and water characteristics (e.g., temperature, light intensity, turbidity) are constantly changing (Crespo 2011; Yee 2016; Chandrasegaran et al. 2018). Similar to the crab *Pachygrapsus marmoratus*, which maintains a high response level to a potential flying predator, mosquito larvae could remain safe by quickly forgetting a previous habituation to a moving object, if this reveals to be innocuous (Tomsic et al. 1993). Similarly to *Neohelice granulata* crabs, previous work by our team has shown that habituation learning in *A. aegypti* larvae is context specific (Pientrantuono et al. 2021). However, here the larvae are not tied to a specific location in their aquatic environment and should not be able to learn differentially to less relevant stimuli over a long period of time.

Second, previous studies have shown that mosquito larvae can perceive a wide range of stimuli, such as light contrast, (Liu et al. 2022), conspecific alarm cues (Ferrari et al. 2008), predator vibrations (Roberts et al. 2019), and predator chemical cues (Chandrasegaran et al. 2018). As part of the neuston, these perceptual abilities, combined with their high speed and their diving reflex, allowed them to escape danger (Mellanby 1958).

Third, when confronted with a repeated stimuli in the same modality, mosquitoes quickly adapt their behaviour in response to experience, i.e., they habituate in few trials. (Baglan et al. 2017; Dessart et al. 2023). Consequently, we can speculate that mosquito larvae should forget quickly and respond strongly to new stimuli after a few times. They should not invest in a costly long-term memory but should favour their own development to minimise time spent in water. This speculation seems interesting when considering the extensive studies on the cognitive abilities of adult mosquitoes, which can retain information for up to 72 hours after visual conditioning (Chilaka et al. 2012) and for which remembering information about the last blood-meal is a crucial adaptative behaviour (Vinauger et al. 2014). Furthermore, while adults *A. aegypti* have been shown to learn to associate an a chemical cue with a blood-reinforced thermal stimulus (Vinauger et al. 2014), the question of the potential ability of mosquito larvae to perform associative learning remains open.

To distinguish habituation to other declines in behaviour, previous authors proposed ten criterions (Rankin et al. 2009). The first criterion define habituation. The second, the spontaneous recovery of the response if the stimulus is withheld, and the eighth, dishabituation, were verified in our previous study (Dessart et al. 2023). By comparing the response after training to several controls, we could rule out sensory adaptation, fatigue, and contextual effects. Here, by increasing the inter-trial interval to 5 min and 10 min, we observed a decrease in individual spontaneous activity during the training. However, the high individual response at the test phase after 24 hours allowed us to rule out all these effects. By increasing the ITI, we also observed that individuals trained with a shorter ITI learned faster than individuals trained with a longer ITI, as revealed by a more rapid decrease in response magnitude along trials at 2 min as compared to others (Figure S1).

This result supports the fourth criterion proposed by Rankin et al. (2009) for describing the behavioural characteristics of habituation, namely that an increase in stimulus frequency leads to an increase in response decrement.

Some other criteria remain to be explored in the habituation of mosquito larvae, for instance the third criterion which states that after repeated sessions of training, the decrement in response becomes more rapid or more pronounced (Rankin et al. 2009). The use of this criterion may explain how larvae can habituate more quickly to new

stimuli shortly after a stimulus has previously appeared, and opens up new questions about the habitat and the cognitive abilities of mosquito larvae.

Finally, the standardised strain reared in the laboratory could influence the ability of *A. aegypti* ability to retain information. Previous authors have shown that a standardized strain could affect the learning abilities and individual fitness (Papaj et al. 1987; Nieberding et al. 2018). Our system is suitable for studying field-reared mosquito larvae and even other small aquatic species that show a comparable escape response.

In this work, we have seen that mosquito larvae have remarkable short-term memory after habituation to an aversive stimulus. This system is well suited to address fundamental questions about larvae abilities to learn and remember, to explore the underlying neurobiological mechanisms, and to ecological perspectives, such as the impact of pollutants or the role of the ecological structure in the development of cognitive abilities.

Acknowledgements

Financial support for this work was granted by Region Centre-Val de Loire, France APR IR 2020 COMPORTATE. COMPORTATE is supported by Réseau MiDi - Milieux et Diversité, Pole DREAM - French Water Team, Zone Atelier de la Loire – LTER France Loire, Réseau ENTOMOCENTRE, Département d'Indre-et-Loire - Direction de l'Attractivité des Territoires (France).

The authors express their gratitude to Joël Meunier, David Baracchi, David Carrasco and Martin Giurfa for fruitful discussions that led to the protocol presented in this paper.

Author contributions

Martin Dessart: Conceptualization**,** Data curation, Formal analysis, Investigation, Methodology, Validation, Software, Visualization, Writing – original draft, Writing – review & editing. **Claudio R. Lazzari:** Conceptualization, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. **Fernando J. Guerrieri:**

Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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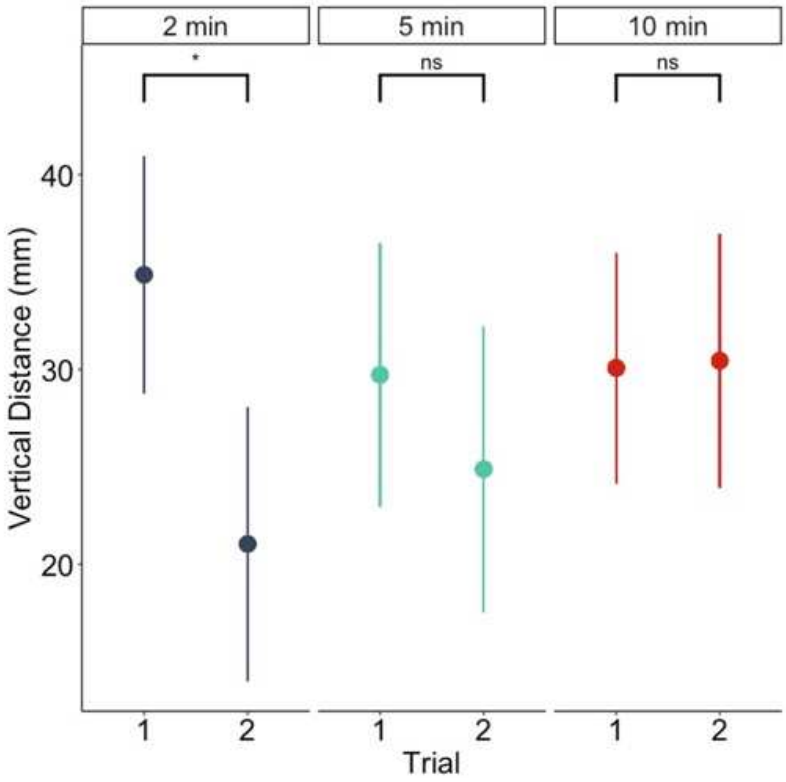
Supplementary material



Supplementary Figure S1: Shorter inter-trial intervals accelerate habituation. For each inter-trial interval, the vertical distance in millimetres travelled by individuals responding to an aversive stimulus during the 1st, and the 2nd trial. 2 min (dark grey), 5 min (green), 10 min (dark red) inter-trial intervals. Points indicate mean values and bars indicate +- 95% confidence intervals. NS, not significant; *P<0.05.

Chapitre 4 : La pollution aigüe et chronique, à doses sous-létales, affecte l'activité, l'apprentissage et la mémoire chez les larves de moustique

Mon beau navire ô ma mémoire
Avons-nous assez navigué
Dans une onde mauvaise à boire
Avons-nous assez divagué
De la belle aube au triste soir

Guillaume **Apollinaire (1913)** La chanson du mal-aimé - *Alcools*

Les **écosystèmes d'eau douce** jouent un rôle essentiel dans le maintien de la biodiversité et fournissent des services écosystémiques essentiels. Pourtant, ils sont de plus en plus menacés par les activités humaines, en particulier la fragmentation des sols, la **pollution** et le changement climatique. Traditionnellement, les méthodes d'évaluation de ces écosystèmes se concentrent sur les propriétés de l'eau. Néanmoins, les approches de **biosurveillance**, en particulier celles qui étudient **le comportement et la cognition**, fournissent des indications précieuses sur l'effet des polluants en contexte écologique. Dans ce chapitre, nous avons étudié l'effet de trois polluants courants (**glyphosate, atrazine** et **paracétamol**) sur les capacités cognitives des larves de moustique ***Aedes aegypti***, vecteur de plusieurs maladies. Nous avons utilisé un dispositif automatisé pour étudier l'apprentissage par **habituation** et les effets des trois polluants, **seuls ou en combinaison**, à des **doses sous létales** allant de doses de terrain à des doses recommandées dans le commerce. Nos résultats montrent que les trois composés **modulent l'activité** spontanée et **altèrent les capacités cognitives** à l'échelle individuelle. Ces changements peuvent altérer la perception ou la réponse comportementale des larves aux signaux de leur environnement, comme la présence de congénères ou de prédateurs, et suggèrent que d'autres organismes vivant dans ces écosystèmes pourraient également être affectés. L'intégration de l'évaluation du comportement et de la cognition en **écotoxicologie** permet de mieux comprendre les effets écologiques des polluants, ce qui est nécessaire afin de relever les défis économiques de ces écosystèmes fragiles.

Ce chapitre a fait l'objet d'une publication au journal : **Journal of Experimental Biology**. L'article est rédigé sous sa forme finale et **en attente de soumission depuis le 13 avril 2024.**

Dessart M, Lazzari C, Guerrieri F (2024) Acute and chronic sublethal chemical pollution affects activity, learning and memory in mosquito larvae.

Acute and chronic sublethal chemical pollution affects activity, learning and memory in mosquito larvae

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Keywords: habituation, *Aedes aegypti*, paracetamol, glyphosate, atrazine, mixture

Highlights

1. We examined the effect of glyphosate, atrazine, and paracetamol, in the cognitive abilities of *Aedes aegypti* mosquito larvae.

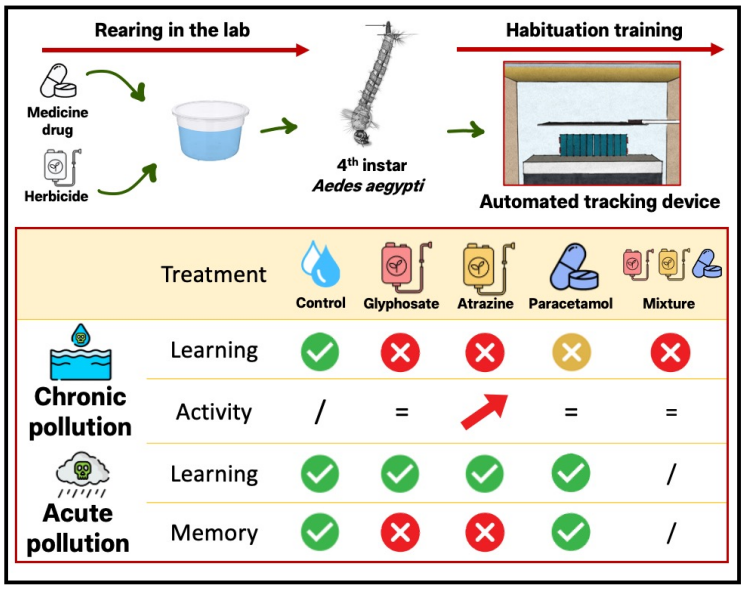
2. We used doses ranging from field-realistic to commercial-recommended concentrations of the pollutants alone and in mixtures.

3. For chronic exposition, spontaneous activity was increased or reduced, and habituation was impaired after pollutant exposure.

4. For acute exposition, memory retention was impaired after pollutant exposure.

5. Using the cognitive abilities of mosquito larvae help to understand the ecological effects of pollutants in vulnerable ecosystems.

Graphical abstract



Abstract

Freshwater ecosystems play a critical role in supporting biodiversity and providing essential environmental services. However, these ecosystems are increasingly threatened by human activities, including habitat loss, pollution, and climate change. Traditional assessment methods focus on water properties, but biomonitoring approaches, particularly those examining behaviour and cognition, provide valuable insights into the ecological effects of pollutants. This study examines the effects of three common pollutants (glyphosate, atrazine, and paracetamol) on the cognitive abilities of *Aedes aegypti* mosquito larvae, a vector for several diseases. We used an automated bioassay to study habituation learning and the effects of the three pollutants alone or in mixtures, at sub-lethal doses ranging from field-realistic to commercial-recommended levels. Our results show that the three compounds modulate individual spontaneous activity, impair habituation and memory retention. These changes may alter the perception or the behavioural response of mosquito larvae to signals of their environment, as the presence of conspecifics or predators, and suggest that other organisms living in freshwater ecosystems may also be affected. Incorporating behavioural and cognitive assessments in ecotoxicological studies provides a more comprehensive understanding of the ecological effects of pollutants which is needed to address economic challenges in fragile ecosystems.

Introduction

Although freshwater ecosystems account for a small proportion of total freshwater (1.3%), which is only 2.7% of the total water on Earth, they provide essential environmental services, support 10% of the world's animal species and are a critical source of biodiversity (Carpenter et al. 2011; Madhav et al. 2020; Zhang et al. 2021). Despite their importance, freshwater ecosystems are the most endangered environments worldwide (Sala et al. 2000; Vári et al. 2022). Threats come from human activities, namely habitat loss and degradation, overexploitation, invasive species, climate change, hydrological alterations and increases in chemical compounds used for industrial, agricultural and domestic purposes (Arthington 2021).

Various methods have been developed to measure changes in these ecosystems. The most common is to take water or sediment samples and measure physical and chemical properties (Bartram et al. 1996; Zhou et al. 2008). In addition, biomonitoring methods have been increasingly explored, ranging from biomarkers at the cellular level to biological indicators or bioindicators at the community level (López-López and Sedeño-Díaz 2015; Zaghloul et al. 2020). These approaches help to address new ecological questions related to ecosystem management and provide tools to target the mechanisms underlying the effects of pollution, to model the toxicity at different intensities and at different temporal and geographical scales (Oertel and Salánki 2003; Previšić et al. 2020; Malhotra et al. 2021). In addition, the multiple sources of toxicity present in the actual environments constantly create unique cocktails, whose effects are difficult to disentangle from individual effects (Hodkinson and Jackson 2005).

At the organism level, studying the effects of toxicity on individual behaviour and cognitive abilities may reveal subtle effects of pollutants that are not accessible to naturalistic observation or standard bioassays (Melvin and Wilson 2013; Bownik and Wlodkowic 2021). In addition, assessing behaviour and cognition in ecotoxicology present other advantages. First, the study of behaviour is more sensitive than community composition or abundance because it integrates physiological processes (Blaxter and Hallers-Tjabbes 1992). Many studies have employed behavioural endpoints to assess the effects of contaminants at sublethal doses which serve as early indicators of

environmental stressors and allow the identification of the mechanisms of action of a toxic response. (Hellou 2011; Hong and Zha 2019). Second, behaviour is linked to individual fitness and is associated with functions such as feeding, anti-predation and reproduction. It is therefore a crucial source of information about the immediate environment, surrounding individuals, and can be used to model population dynamics (Ford et al. 2021). Third, the incorporation of behavioural and cognitive bioassays into ecotoxicological assessment links laboratory studies to more ecologically relevant scenarios (Bertram et al. 2022). Fourth, behavioural adaptations or maladaptations to pollutants provide insight into potential evolutionary changes that may occur within an ecosystem (Jacquin et al. 2020). Finally, despite the lack of standardised methods, behavioural bioassays are easy to perform, non-invasive, inexpensive and have a precise ecological relevance (Bonada et al. 2006).

For example, a recent paper by Li et al. (Li et al. 2019) examined the role of lead at field-realistic concentrations on the behaviour and physiology of the zebrafish *Danio rerio*. First, they assessed the cognitive abilities and physiological alterations of individuals exposed to three concentrations of lead and of a control group. Then, they generated *behavioural fingerprints*. Behavioural fingerprints are the result of a combination of behavioural parameters applied for comprehensively measuring neurotoxic effects (Li et al. 2019). Finally, they measured the expression of mRNA levels and performed histopathological analysis of brain tissue. Within a comprehensive behavioural and physiological analysis, the authors provided a global approach to study subtle effects of lead in behaviour and physiology, as well as ecological interpretations (Li et al. 2019).

In this study, we examined the effects of three pollutants adjusted for acute and chronic toxicity, ranging from doses measured directly in water to spray doses usually recommended for agricultural or gardening use, alone or in combination, on the locomotor, sensory, learning and memory abilities of dengue mosquito larvae. *Aedes aegypti* is the most cosmopolitan disease vector insect, responsible for the transmission of the causative agents of numerous human an animal diseases, including Dengue fever, Chikungunya fever, Zika virus, yellow fever, West Nile virus and Dirofilariasis (Kraemer et al. 2019; Adegoke et al. 2020). Due to the use of insecticides worldwide, a selective pressure has favoured the development of resistance mechanisms in this species, which

are currently under investigation (Rubio-Palis et al. 2023). Exposure of *Aedes aegypti* larvae to atrazine or glyphosate increases the expression of cytochrome P450 monooxygenases (P450s), glutathione-Stransferases (GSTs), and carboxylesterases that can confer larval tolerance to different insecticides (Bara et al. 2014).

The larval stage, which lasts about 4 to 10 days, which is easy to rear and rapidly developing in the laboratory is, in addition, sensitive to pollution (Boyer et al. 2006; Baglan et al. 2018; Black et al. 2021). Mosquito larvae spend most of their time just below the surface of the water. Larvae dive when they perceive potential threat, for example, a moving shadow over the water or a vibration (Clements 1999). If the stimulus is found to be harmless after repeated occurrences, the larvae become habituated to it, namely, they gradually reduce their response to further stimulation (Baglan et al. 2017). The stimulus perceived as dangerous by naïve animals is no longer significant in experienced individuals. We adopted a habituation (a form of non-associative learning) training procedure by Dessart et al. (2023) the basis for a bioassay to evaluate the effects of pollutants on cognitive abilities and behaviour in mosquito larvae (Dessart et al. 2024).

Here, we investigated the effects of two herbicides (atrazine and glyphosate) and a medicinal drug (paracetamol) on habituation in *Aedes aegypti* larvae. Glyphosate is the active ingredient in herbicide formulations and the most heavily used agrochemical in the world (Battaglin et al. 2014; Gill et al. 2018). It can persist in soils for years and is commonly found in aquatic ecosystems (Kanabar et al. 2021). It has been shown to impair collective thermoregulation and learning in bumblebees, and to alter life history traits, nutritional stress, and learning in mosquitoes (Bara et al. 2014; Baglan et al. 2018; Bataillard et al. 2020; Weidenmüller et al. 2022; Nouvian et al. 2023).

Atrazine is another widely used herbicide in the world (Li et al. 2018). It has been banned in the EU since 2004 but remains the most commonly used herbicide in the USA and is often found in water (Bara et al. 2014; Abdulelah et al. 2020).

The effects of atrazine have been studied on the locomotor activity in mammals, amphibians and teleost fishes, honeybees and nematodes (Rohr and McCoy 2010; García-Espiñeira 2018; Araújo et al. 2021), life history traits in mosquitoes (Bara et al. 2014), and spatial learning and memory in mice (Rastegar-Moghaddam et al. 2019). Paracetamol (acetaminophen) is the active ingredient in pharmaceutical products used as analgesics and is the most widely used drug worldwide (Duong et al. 2016; Hider-

Mlynarz et al. 2018; McCrae et al. 2018). Bioassays investigating the effects of paracetamol on behaviour are scarce, but a study in the zebrafish *Danio rerio* showed toxic effects of paracetamol on individual malformations, pigmentation, locomotion, enzyme expression and epigenetics (Nogueira et al. 2019).

In this study, we performed a series of bioassays using each substance alone at different concentrations or in mixtures.

Material and methods

1. Animals

Mosquito eggs (*Aedes aegypti* Bora strain) were obtained from MIVEGEC-IRD (Montpellier, France). Eggs were kept dry or placed in 750 ml polypropylene containers with either 500 ml dechlorinated tap water or 500 ml mixture. Larvae were fed *ad libitum* with shrimp food (JBL Novo Prawn, Neuhofen, Germany) and the containers were maintained at 25°C ± 2°C, under a 12h:12h light/dark cycle (lights on at 8:00 am). Only fourth instar mosquito larvae were trained in the experiments. All animals were reared and manipulated in accordance with European Union ethical guidelines.

2. Experimental procedure

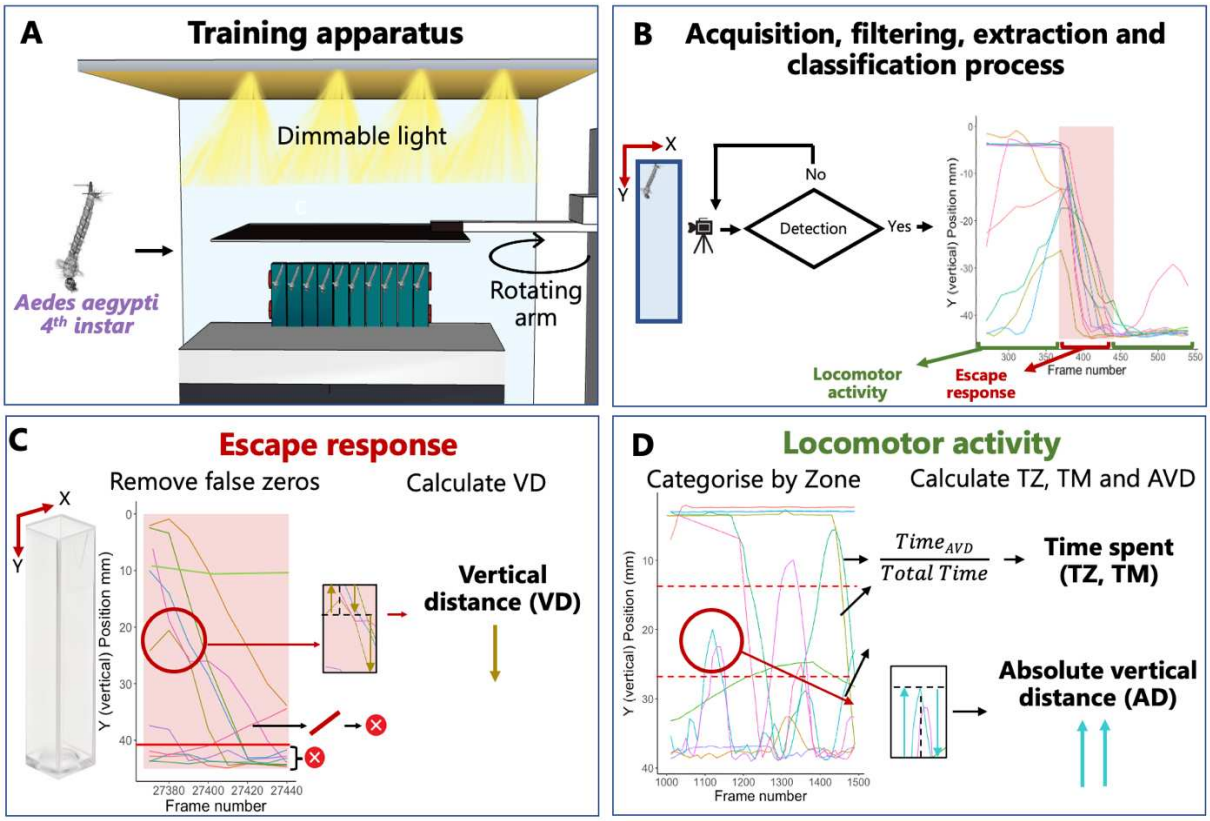


Figure 1: Schematic of the experimental protocol. A) *Aedes aegypti* larvae were reared in the laboratory and trained at the fourth larval stage using our training apparatus. B) Experiments were video-recorded and individual trajectory was extracted. C) We analysed the stimulation response that corresponded to the individual trajectory during the aversive stimulus, using Vertical distance (VD). This quantitative variable was calculated as the relative sum of the distance travelled in the vertical direction toward the bottom of the cuvette. In addition, two filters were applied to exclude individuals located at the bottom of the cuvette during the first frames of the aversive stimulus and the individuals travelling upwards during the stimulus. D) We analysed the spontaneous locomotor activity using 3 variables. Time spent per zone (TZ) was a proportion of time spend in one of the 3 zones delimited. Time spent moving (TM) was a proportion of time where the Absolute vertical distance was above a threshold of 1mm/sec. Absolute vertical distance (AD) was quantitative and calculated as the absolute sum of the distance travelled in the vertical direction.

All experiments were performed in the same experimental room at 25°C ± 0.5°C during the afternoon (i.e., from 12.00 to 19.00 h). The experimental apparatus was the same used in two previous studies and consisted of a platform for isolating individual mosquito larvae, different types of stimuli, and a system for recording and analysing larval behaviour (Dessart et al. 2023, 2024). It included individual containers (i.e., 1.5 ml spectrophotometer plastic cuvettes) filled with treated water (see acute toxicity section below) or dechlorinated tap water (Figure 1A). Fourth instar larvae were placed in 10 horizontally aligned cuvettes and confronted with the appearance of an aversive stimulus consisting of either a black cardboard square (16 cm side) attached to a

servomotor, or 4 mechanical vibrators, all operated by an Arduino Uno board remotely controlled by a computer. The cardboard represented a potential flying predator visually perceived by the larvae, while the vibrator represented the motion of potential fishes transmitted through the water column. The cuvettes were illuminated by two LED panels and the light intensity was fixed at 900 $\mu W.cm^{-2}$ ± 100 $\mu W.cm^{-2}$ (International Light Technology radiometer). All experiments were video-recorded, and the videos were subsequently analysed (Figure 1B, data analysis section).

After 30 min of familiarisation, 10 trials were performed, i.e., 10 passages of the visual stimulation, with a 2 min inter-trial interval. For the acute toxicity (see acute toxicity section), a new trial was performed 3 hours after the 10^{th} trial to test the memory persistence of mosquito larvae. After training, we applied a 3-second mechanical stimulation using the vibrators to check that the larvae were still responsive to a stimulus. Individuals that did not respond to the mechanical stimulus were excluded from the analysis (7 individuals). We also removed individuals that remained motionless during the training (10 individuals), transformed into pupae during the training (1 individual), and when tracking failed to extract individual coordinates (1 individual). A total of 765 individuals were kept for the analyses.

3. Chemical treatment

Two types of toxicity were represented in this study. Chronic toxicity represents the effect of exposure at low concentrations over the development from egg to 4^{th}- instar, whereas acute toxicity was achieved by a single exposure to higher concentrations of chemicals (Environmental Protection Agency 1994). We simulated chronic toxicity by placing eggs in 750 ml polypropylene containers with either 500 ml of water purified using a Millipore Milli-Q lab water system or 500 ml of treated water, until the fourth larval stage. To represent acute toxicity, larvae were reared in 500 ml purified water, and they were subsequently trained in treated water or purified water (during 30 min familiarisation + training period). Three chemicals were considered for both toxicity simulations: glyphosate, atrazine and paracetamol. Glyphosate solutions were prepared by dissolving glyphosate (PESTANAL analytical standard, purity ≥98%, Sigma Aldrich, USA), atrazine (PESTANAL analytical standard, purity ≥98%, Sigma Aldrich, USA) and paracetamol (BioXtra, purity ≥98%, Sigma Aldrich, USA) in Millipore Milli-Q lab water. We

used the pure chemical rather than a formulation to isolate any possible effect due to these substances being in a mixture. Indeed, formulations with several compounds have been shown to be more toxic than the chemical alone (Nagy et al. 2020).

4. Chronic toxicity

To investigate the chronic toxicity of the two herbicides (glyphosate and atrazine) and the medicine drug (paracetamol), mosquito larvae were reared in treated water. For glyphosate, we selected two concentrations related to field measurements: 100 µg/L and 200 µg/L (Struger et al. 2008; Byer et al. 2008; Riaz et al. 2009) and a high concentration was prepared at 2mg/L which corresponds to usual doses applicated in other works (Bara et al. 2014; Balbuena et al. 2015; Baglan et al. 2018; Nouvian et al. 2023). For atrazine, three concentrations were selected: 200 µg/L and 500 µg/L and 2mg/L, consistently with several publications (Dewey 1986; Bara et al. 2014; Johnson 2019; Abdulelah et al. 2020; Adedara et al. 2021). For paracetamol, although field concentrations reported by some studies were relatively low (e.g. (Gracia-Lor et al. 2012): median 44.8 µg/L, min–max values 1.13 – 201 µg/L), we chose to test concentrations of 1 mg/L, 10 mg/L and 100 mg/L in line with previous bioassays in mammals (Adamson et al. 1991; El Menyiy et al. 2018), the zebrafish *Danio rerio* (Nogueira et al. 2019) and on the development of the fly *Calliphora vicina* (O'Brien and Turner 2004).

A control treatment was paired with each set of conditions. A first control (N°1) was associated with atrazine 200 µg/L and 500 µg/L, and a second control (N°2) was associated with glyphosate 100 µg/L and 200 µg/L, and with paracetamol 1 mg/L and 10 mg/L. This first experiment was called "field doses" because it represented low concentrations associated with field measurements. A new control (N°3) was associated with atrazine 2 mg/L and another one (N°4) with glyphosate 2 mg/L and paracetamol 100 mg/L. This second experiment was called "spray doses" because it represented high concentrations associated with field applications.

In a second set of experiments, we compared the effect of chronic toxicity of chemicals alone or in mixtures alongside the control treatment. The first mixture consisted of concentrations measured directly in water, in our region. A paracetamol concentration of 6 µg/L corresponded to the maximum measured in the Loire River basin

between 2018 and 2019 (Ledieu et al. 2021); the concentrations of 0.126 µg/L for atrazine, and 2.3 µg/L for glyphosate corresponded to the highest concentrations found at monitored sites in the Indre et Loire area between 2019 and 2021 (www.naiades.eaufrance.fr). These concentrations were communicated by the Institute of Organic and Analytical Chemistry (ICOA UMR 7311 CNRS – *Université d'Orléans*).

The second mixture consisted of 200 µg/L of atrazine and 1mg/L of paracetamol, two treatments that did not affect individual learning (see Results section below). A control (N°5) was associated with the chemicals alone, and an additional control (N°6) was associated with the two mixtures, in this set of experiments called "field realistic doses" *sensu* Herbert et al. (Herbert et al. 2014).

5. Acute toxicity

To assess acute toxicity, we reared larvae in purified water and trained them in treated water. We chose the highest concentrations similar to those used in the chronic toxicity tests (glyphosate and atrazine: 2 mg/L, paracetamol: 100 mg/L). We analysed the effect of these treatments on individual learning, and we also investigated whether these treatments would affect individual memory retention by applying a new trial 2 h after the training session. This retention time was chosen based on our previous work showing that larvae could retain information for up to 2 h after visual learning (Dessart et al. 2023). A last control (N°7) was associated with these experiments.

6. Data analysis

For each experiment of 10 individuals, two sets of videos were recorded. First, we recorded the familiarisation during 30 min and compared global spontaneous activity between groups by taking the last 5 min of the familiarisation. Then, we recorded the whole duration of the training period. The videos were analysed similarly as in Dessart et al. (2024). Briefly, each individual trajectory was extracted using a tracking software and larval identity and detection rate were verified (Figure 1B); Supplementary Table T1, Supplementary Table T2). Individual vertical positions were smoothed and collapsed to reduce the data number, they were classified into two categories and analysed separately (Figure 1). First, the escape response lasted for the 3 seconds period during which the visual stimulus appeared above the individuals (Figure 1C). These data were

filtered within each trial response to remove false zeros (i.e., when the larvae were already at the bottom of the cuvette at the start of a trial, see [34]). After this step, we defined the vertical distance (VD) as the response variable corresponding to the escape response. This variable was 0 if the individual did not move during a stimulus period, and increased as the individual moved away from the stimulus (i.e., dived downwards, Figure 1C). Second, locomotor activity represented individual spontaneous activity during the familiarisation and the inter-trial interval period (Figure 1D). These data were outside the stimulus period and were therefore not filtered. We analysed locomotor activity using the absolute distance (AD), which measured individual displacement irrespective of the direction of movement (upward or downward). This variable was more consistent in representing the average speed and number of diving events of an individual across the ITIs. Finally, we also divided the cuvette in three equal zones (Figure 1D) and calculated the time spent per zone (TZ) and the time spent moving (TM).

Statistical analyses

7. Learning performance

We modelled the learning performance using the Generalised Additive Model to provide a visual estimate of the behavioural response across trials during the training period. We defined models of increasing complexity and compared the best smoothing function using the GCV-UBRE criterion from the *mgcv* package (Wood 2017). Similar to our previous work, the best smoothing function was the P-spline and was applied to all smoothing curves (Dessart et al. 2024).

For each treatment, we compared the distance travelled during a stimulation on the first and last trials. If the difference was not statistically significant, we concluded that the individuals had not habituated. To make these comparisons, we applied a linear mixed effects model to compare the response at the 1st trial versus the 10th trial. The control treatments were analysed similarly to verify that the individuals reared and trained in clear water were able to habituate. We chose VD as the response variable, trial as a fixed factor and the individual identity as a random factor. We checked variance homogeneity and the distribution of the residuals using the *simulateResiduals* function from the *DHARma* package (Hartig F 2022). For the assessment of acute toxicity, we

applied the same model but including the response at the Test phase. For these models, we evaluated pairwise comparisons using the *emmeans* package with Tukey correction (Lenth 2021).

8. Spontaneous activity

We compared individual spontaneous activity during familiarisation, considering the last 5 min of each treatment. We also compared the 9 inter-trial intervals (ITI) between the treatments. First, the absolute distance (AD) travelled by individuals was averaged to calculate the individual average speed (mm/sec) (Figure 1D). We divided the cuvette into three equal zones (top, middle, bottom, Figure 1D), and counted the time spent in each zone (%). We also confronted AD with a threshold of 1mm/sec and classified our data into the variable time spent moving (Figure 1D). Furthermore, we counted the number of diving events by creating a function that counted each time an individual crossed 1/3 and 2/3 of the cuvette length (i.e. 120 pixels) on its way in and out (Figure 1D). For the average speed, the time spent moving and the number of diving events comparison, we applied a one-way ANOVA with respectively AD, time spent moving or the diving events as the response variable and the treatment as factor, followed by Tukey's post hoc test for multiple comparisons.

9. Dataset and code repository

The R code used to analyse the data and the database are available online at: https://github.com/martindessart/Chemical_toxicity

Results

1. Chronic exposition to field doses

Learning

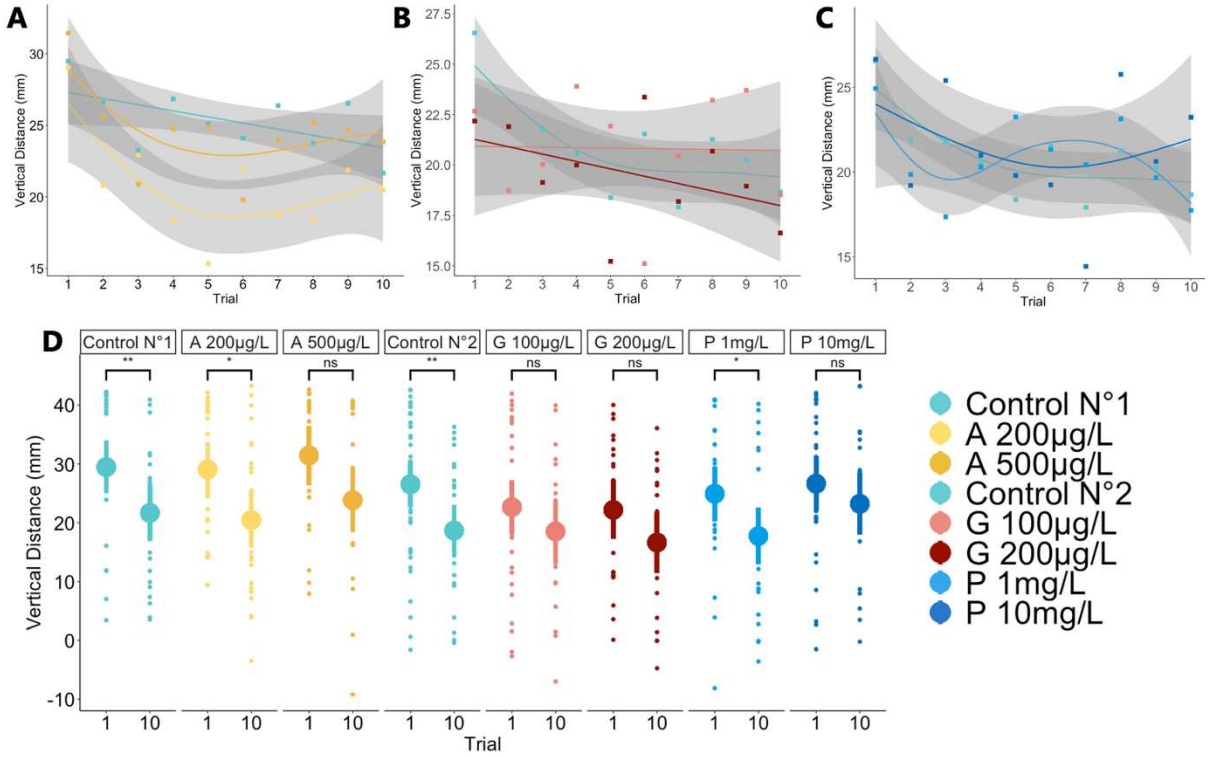


Figure 2: Learning performance for larvae reared at field doses. A) B) C) Habituation curves for larvae reared in control (cyan), atrazine 200 µg/L (light yellow), atrazine 500 µg/L (dark yellow), glyphosate 100 µg/L (pink), glyphosate 200 µg/L (red), paracetamol 1 mg/L (light blue), paracetamol 10 mg/L (dark blue). Vertical distance (in millimetre) corresponds to the distance travelled by one individual during the stimulus period (3 sec), from the 1st to the 10th trial. Smoothing lines indicate the best fitted GAM model. Grey shades indicate 95% confidence interval for the average response. Points indicate mean values. D) For each treatment, vertical distance in millimetre travelled by individuals responding to an aversive stimulus during the 1st to the 10th trial. Points indicate mean values and bars indicate +- 95% confidence intervals. A= Atrazine, G = Glyphosate, P = Paracetamol. NS, not significant; *P<0.05, ***P<0.001.

For control N°1, control N°2, atrazine at 200 µg/L and paracetamol at 1 mg/L, VD was significantly higher in the 1st trial than in the 10th trial: Control N°1: t-ratio = 3.373, df =27, P < 0.01; A 200 µg/L: t-ratio = 2.631, df =28, P = 0.01, Control N°2: t-ratio = 2.801, df =39, P < 0.01; P 1 mg/L: t-ratio = 2.618, df =25, P = 0.01 (Figure 2). On the opposite, the difference between the 1st and the 10th trial was not significant for the other treatments: A 500 µg/L: t-ratio = 1.974, df =23, P = 0.06; G 100 µg/L: t-ratio = 1.059, df =25, P = 0.30; G 200 µg/L: t-ratio = 0.243, df =22, P = 0.81; P 10 mg/L: t-ratio = 1.408, df =22, P = 0.173 (Figure 2).

Activity

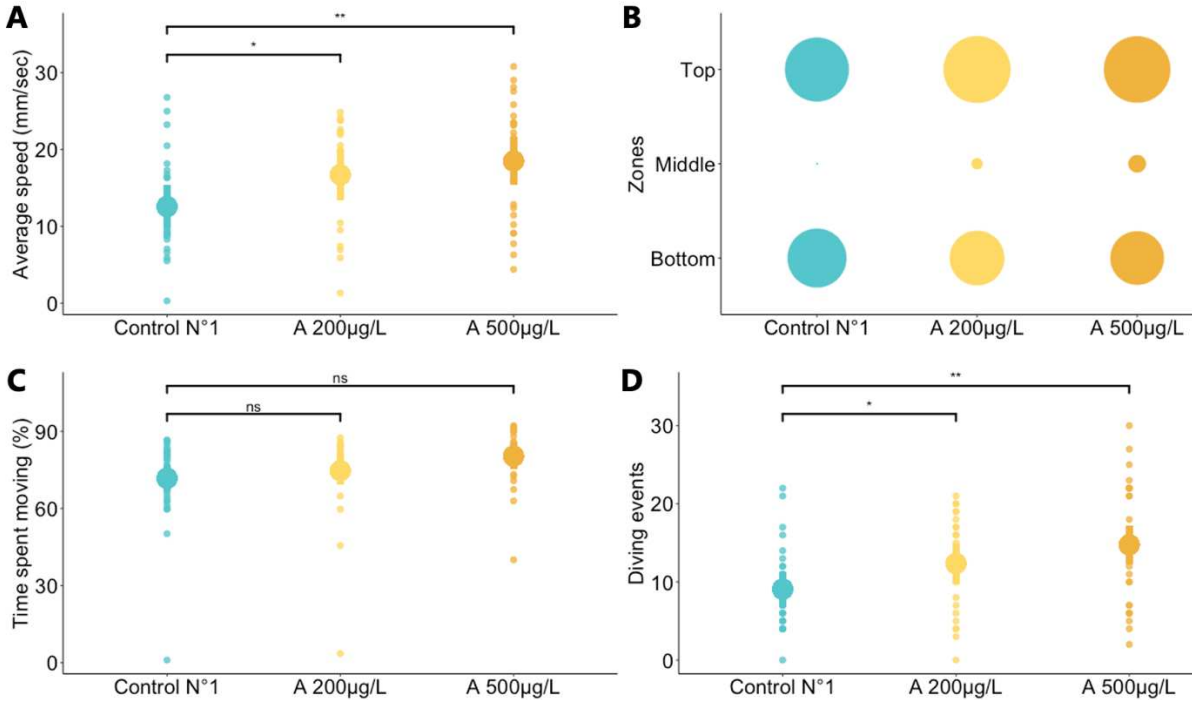


Figure 3: Example of spontaneous locomotor activity for larvae reared in atrazine at field doses during familiarisation. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. A = Atrazine. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant; *P<0.05, **P<0.01.

All the results of spontaneous activity are detailed in supplementary material (Supplementary Table T4). For the treatments at field doses, individuals reared in atrazine were faster and dived more often than control, both during the familiarisation and during the inter-trial intervals (Average speed: Familiarisation: A200 µg/L: t-ratio = -2.427, df =85, P = 0.05; A500 µg/L: t-ratio = -3.593, df =85, P = < 0.01; Inter-trial intervals: A500 µg/L: t-ratio = -2.603, df =90, P = 0.03; Diving events: Familiarisation: A200 µg/L: t-ratio = -1.99, df =85, P = 0.05; A500 µg/L: t-ratio = -3.565, df =85, P = < 0.01; Inter-trial intervals: A500 µg/L: t-ratio = -2.755, df =90, P = 0.01; Figure 3). All other comparisons were not significant (Supplementary Table T4, Supplementary Figure S1, S2, S3, S9, S10).

2. Chronic exposition to spray doses

Learning

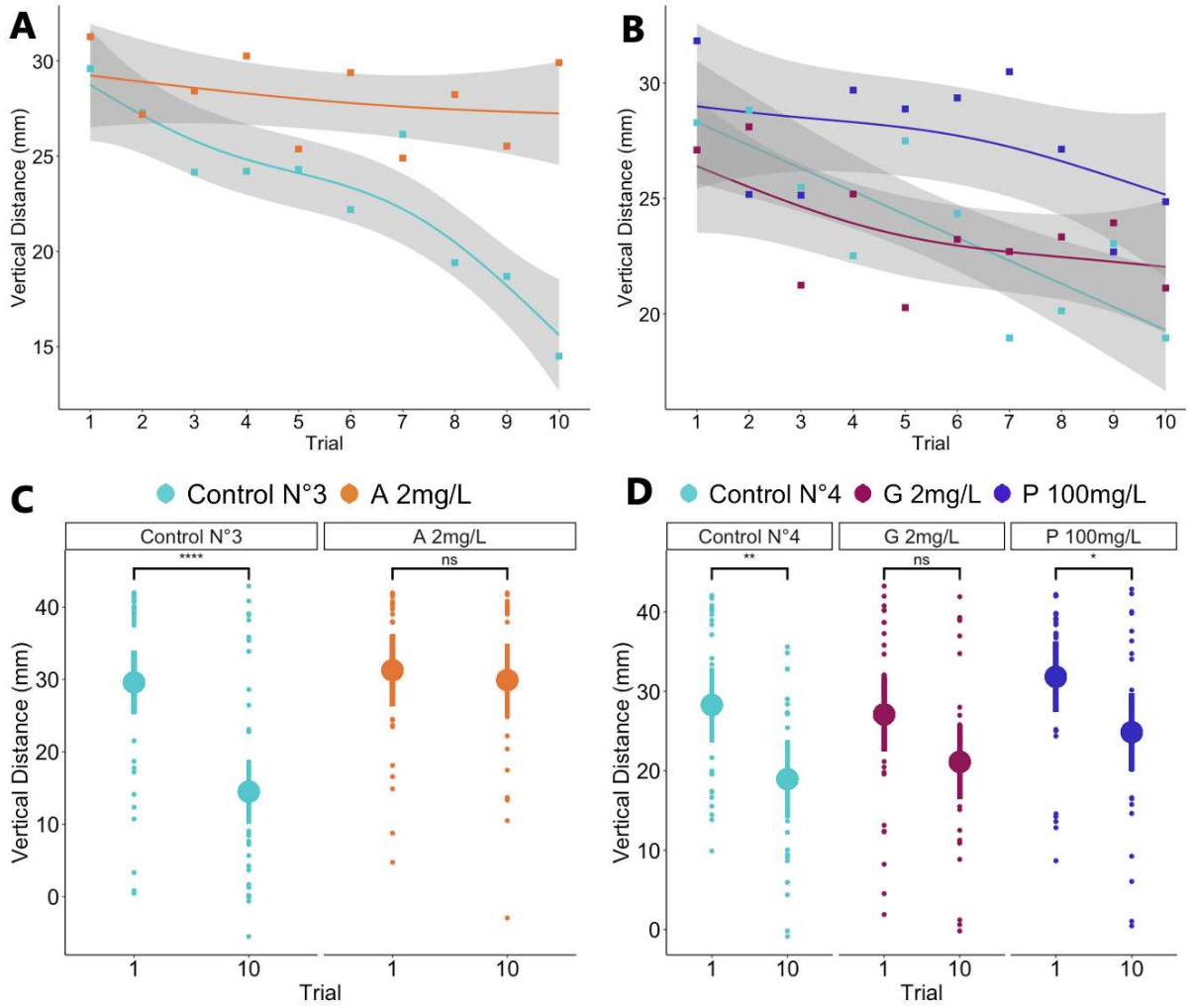


Figure 4: Learning performance for larvae reared at spray doses. A) B) Habituation curves for larvae reared in control (cyan), atrazine 2 mg/L (orange), glyphosate 2 mg/L (purple), paracetamol 100 mg/L (dark blue). Vertical distance (in millimetre) corresponds to the distance travelled by one individual during the stimulus period (3 sec), from the 1st to the 10th trial. Smoothing lines indicate the best fitted GAM model. Grey shades indicate 95% confidence interval for the average response. Points indicate mean values. C D) For each treatment, vertical distance in millimetre travelled by individuals responding to an aversive stimulus during the 1st to the 10th trial. Points indicate mean values and bars indicate +- 95% confidence intervals. A= Atrazine, G = Glyphosate, P = Paracetamol. NS, not significant; *P<0.05, **P<0.01, ****P<0.0001.

For the controls N°3 and N°4 and paracetamol at 100 mg/L, VD was significantly higher in the 1st trial than in the 10th trial: Control N°3: t-ratio = 4.686, df =35, P < 0.0001; Control N°4: t-ratio = 3.063, df = 23, P < 0.01; P: t-ratio = 2.676, df = 25, P = 0.01 (Figure 4). On the opposite, the treatments with atrazine and glyphosate at high dose did not present a significant difference: A 2 mg/L: t-ratio = 0.443, df =27, P = 0.66; G 2 mg/L: t-ratio = 1.761, df = 25, P = 0.09 (Figure 4).

Activity

Larvae reared in glyphosate at 2 mg/L and paracetamol at 100 mg/L dived more than control (Familiarisation: P 100 mg/L: t-ratio = -2.906, df = 83, P = 0.01; Inter-trial intervals: G 2 mg/L: t-ratio = -4.622, df = 83, P < 0.0001; P 100 mg/L: t-ratio = -4.344, df = 83, P < 0.001; Figure 8). Larvae reared in glyphosate were also faster than control during the inter-trial intervals (Average speed G 2 mg/L: t-ratio = -3.033, df = 83, P < 0.01). All other comparisons were not significant (Supplementary Table T4, Supplementary Figure S4, S5, S11, S12).

3. Chronic exposition to field realistic doses

Learning

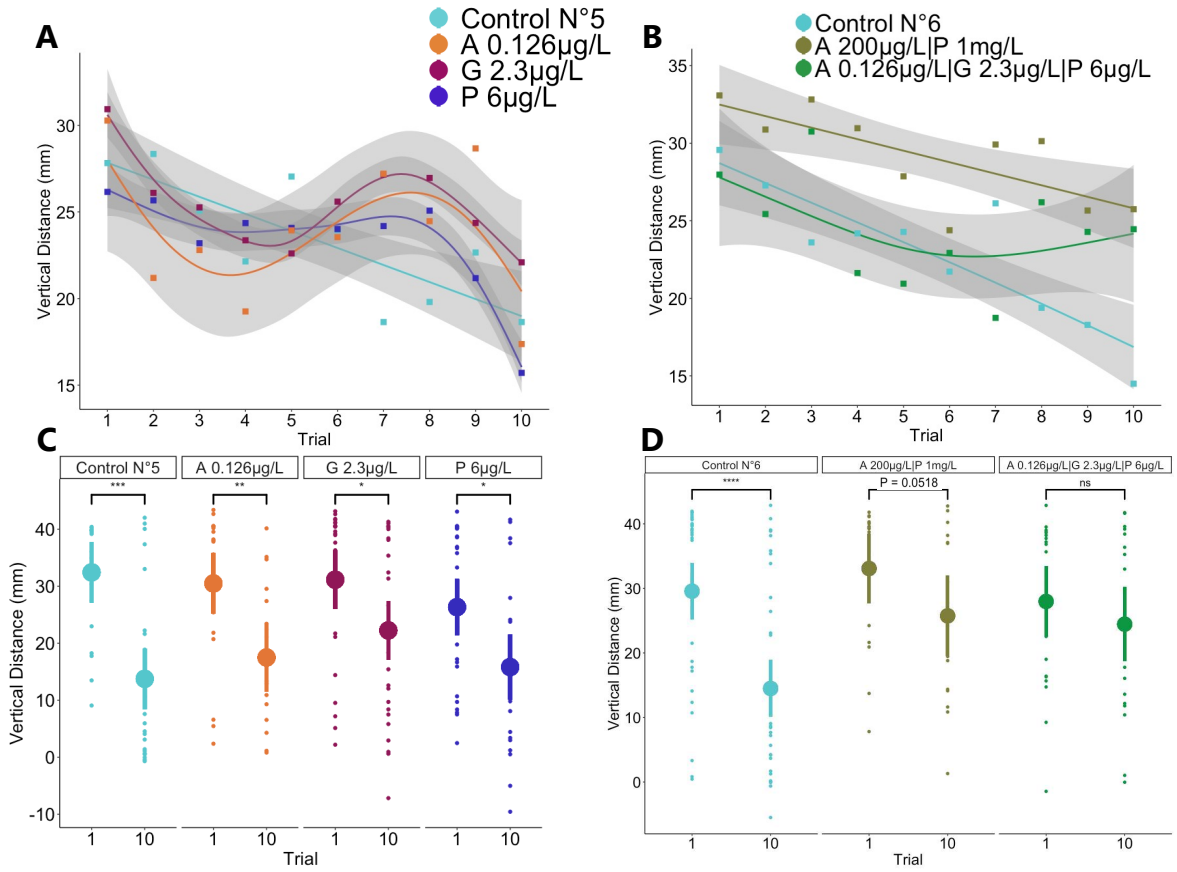


Figure 5: Learning performance for larvae reared at realistic doses. A) B) Habituation curves for larvae reared in control (cyan), atrazine 0.126 µg/L (orange), glyphosate 2.3 µg/L (purple), paracetamol 6 µg/L (dark blue), Mixture N°1 (light green), Mixture N°2 (dark green). Vertical distance (in millimetre) corresponds to the distance travelled by one individual during the stimulus period (3 sec), from the 1st to the 10th trial. Smoothing lines indicate the best fitted GAM model. Grey shades indicate 95% confidence interval for the average response. Points indicate mean values. C) D) For each treatment, vertical distance in millimetre travelled by individuals responding to an aversive stimulus during the 1st to the 10th trial. Points indicate mean values and bars indicate +- 95% confidence intervals. A= Atrazine, G = Glyphosate, P = Paracetamol. NS, not significant; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

For the controls N°5 and N°6 and the chemicals alone, VD was significantly higher in the 1st trial than in the 10th trial: Control N°5: t-ratio = 5.512, df = 24, P < 0.01; A: t-ratio = 4.301, df = 21, P < 0.001; G: t-ratio = 2.205, df = 27, P = 0.04; P: t-ratio = 2.729, df = 26, P = 0.01; Control N°6: t-ratio = 4.686, df = 35, P < 0.001 (Figure 5). However, for the mixture N°1, the difference was not significant: t-ratio = 0.959, df = 22, P = 0.35. Finally, we found a marginal difference for the mixture N°2: t-ratio = 2.058, df = 22, P = 0.0518 (Figure 5).

Activity

Larvae reared in 6 µg/L of paracetamol dived significantly less than control N°5 during inter-trial intervals only (P 6 µg/L: t-ratio = -2.789, df =113, P = 0.03 (Figure 10). Regarding mixtures, larvae reared in the mixture N°2 were faster and dived significantly more than control N°6 during familiarisation and inter-trial intervals (Average speed: Familiarisation: Mixture N°2: t-ratio = -1.988, df =89, P = 0.05; Inter-trial intervals: Mixture N°2: t-ratio = -3.053, df =89, P < 0.01; Diving events: Familiarisation: Mixture N°2: t-ratio = -3.64, df =89, P < 0.01; Inter-trial intervals: Mixture N°2: t-ratio = -3.602, df =89, P < 0.01; Figure 11). All other activity showed no difference between the controls and the treatments (Supplementary Table T4, Supplementary Figure S6, S7, S13, S14).

4. Acute toxicity

Learning and memory

For the four treatments, VD was significantly higher in the 1st trial than in the 10th trial: Control N°7: t-ratio = 2.704, df = 49, P = 0.03; A: t-ratio = 2.801, df = 42, P = 0.02; G: t-ratio = 3.118, df = 53, P < 0.01; P: t-ratio = 4.002, df = 51, P < 0.001 (Figure 6). To evaluate the duration of memory of the larvae tested after 2 hours, we compared the response at the 1st trial to the response at the Test phase. While the difference was significant for Control and paracetamol (Control N°7: t-ratio = 2.906, df = 45, P = 0.01; P: t-ratio = 3.454, df = 52, P < 0.01), it was not the case for atrazine and glyphosate (A: t-ratio = 1.612, df = 41, P = 0.25; G: t-ratio = 0.612, df = 53, P = 0.815, Figure 6).

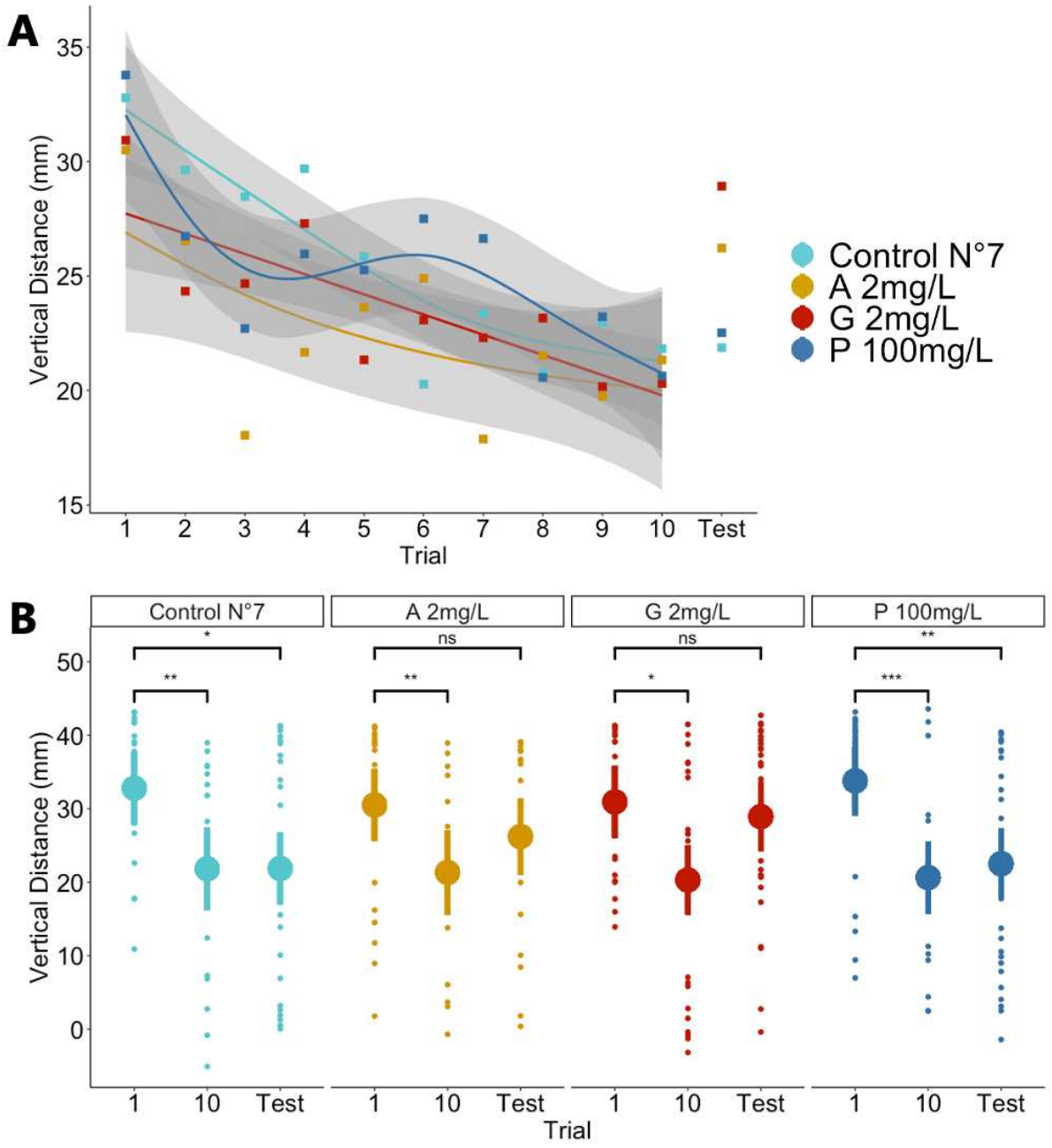


Figure 6: Learning performance for larvae reared at spray doses for acute toxicity. A) Habituation curves for larvae reared in control (cyan), atrazine 2 mg/L (yellow), glyphosate 2 mg/L (red), paracetamol 100 mg/L (blue). Vertical distance (in millimetre) corresponds to the distance travelled by one individual during the stimulus period (3 sec), from the 1st to the 10th trial. Smoothing lines indicate the best fitted GAM model. Grey shades indicate 95% confidence interval for the average response. Points indicate mean values. B) For each treatment, vertical distance in millimetre travelled by individuals responding to an aversive stimulus during the 1st to the 10th trial. Points indicate mean values and bars indicate +- 95% confidence intervals. A= Atrazine, G = Glyphosate, P = Paracetamol. NS, not significant; *P<0.05, **P<0.01, ***P<0.001.

Activity

Larvae trained in paracetamol at 100 mg/L showed reduced time spent moving both during the familiarisation and the inter-trial intervals (Familiarisation: P 100 mg/L: t-ratio = 3.008, df = 114, P = 0.02; Inter-trial intervals: P 100 mg/L: t-ratio = 2.760, df = 112, P = 0.03; Figure 13). All other comparisons showed no difference in spontaneous activity (Supplementary Table T4, Supplementary Figure S8, S15).

Discussion

The development of behavioural tests to assess the effects of pollutants at field realistic concentrations on the cognitive abilities of animals provides an understanding of how pollutants may affect higher neurological functions as well as their environment (Bownik and Wlodkowic 2021). This study investigated how three pollutants at field realistic concentrations affect the activity, learning and memory in mosquito larvae. Our results show that atrazine, glyphosate and paracetamol can reduce or increase individual spontaneous activity, impair habituation and memory retention 2 h after exposure to the pollutants. These changes the animals' ability to perceive and escape from a potential danger or to avoid spending energy escaping from an innocuous object in its natural habitat. In result, it may reduce the individuals' overall fitness. Our results put into evidence behavioural changes due to sub-lethal doses of pollutants in the water. Therefore, mosquito larvae could potentially be used as bioindicators to study the effect of chemicals at sub-lethal doses. Moreover, we observed deleterious effects of glyphosate, atrazine and paracetamol presented in a mixture, at doses in which no effect could be observed due to each substance alone. This means that our bioassay proved to be useful to put into evidence toxic effects of the mixture itself also known as "cocktail effects", which cannot be predicted solely by chemical analyses.

In this study, given that we were interested on sub-lethal hidden effects, we did not perform a survival analysis, as was previously done for glyphosate (Baglan et al. 2018). Although we cannot state that individual survival was not impacted at all, we did not observe any noticeable mortality during the rearing of the larvae. In the study by Baglan et al. (Baglan et al. 2018), the authors used glyphosate concentrations similar to

ours, and no reduction in survival was assessed. In addition, a study by Bara et al. (Bara et al. 2014) examined the effects of atrazine and glyphosate at 5 mg/L on life history traits in *Aedes aegpyti* and *Aedes albopictus* mosquitoes. Atrazine increased the emergence rate and the emergence time of *Aedes aegpyti*, and also skewed the sex-ratio. Another study (Rakotondravelo et al. 2006) assessed the sublethal effects of atrazine on survival, growth and adult emergence of the aquatic midge *Chironomus tentans*, and no effect on these three parameters at 150 □g/L was found. For the cockroach *Nauphoeta cinerea,* (Adedara et al. 2021) no effect on individual survival was found when cockroaches had been administered 1.0 and 0.5 µg g^{-1} of atrazine. Finally, Marcus et al. (Marcus and Fiumera 2016) found a reduction in survival in the fly *Drosophila melanogaster* starting at 2 ppB of atrazine dissolved in the diet. To the best of our knowledge, we did not find any studies on the effect of paracetamol on mosquito survival, and we did not observe any specific mortality in our study.

Atrazine affected our animals by altering their learning ability and by increasing their spontaneous locomotion at 500 □g/L. At 200 □g/L, atrazine slightly increased individual spontaneous activity but not at 2 mg/L. At the latter concentration, however, learning ability was severely impaired, as the average distance travelled during a stimulus was not less than 25 mm, whereas it fell below 20 mm in the control treatments. Furthermore, when looking at the response 2 h after the end of the training phase (i.e., acute toxicity), atrazine impaired the individual's ability to retrieve the information, whereas learning was not impaired. These three independent results provided strong evidence for the neurotoxicity of atrazine in mosquito larvae. As noted by several authors, atrazine disrupts endocrine functions in invertebrates, by increasing oxidative stress and increased the activity of enzymes like P450 known to be involved in insecticide resistance in mosquitoes (Semren et al. 2018). Moreover, atrazine has been shown to alter acetylcholinesterase activity in invertebrate species (Boyer et al. 2006; Vogel 2015).

Besides, atrazine induced changes in locomotion, still with conflicting results. For instance, it decreased locomotor activity in cockroaches, honeybees, nematodes and termites (García-Espiñeira 2018; Ejomah et al. 2020; Araújo et al. 2021; Adedara et al. 2021), while in amphibians it increased activity at low concentrations, and decreased activity at high concentrations (Rohr and McCoy 2010). In our study, we hypothesise that the hyperactivity induced at field concentrations was attenuated by greater toxicity at

higher concentrations. To our knowledge, the only studies of the effects of atrazine on learning and memory have been in mammals. For example, Rastegar-Morghaddam et al. (Rastegar-Moghaddam et al. 2019) found that atrazine ingestion impaired learning and spatial memory in mice and increased the apoptosis of cells located in the hippocampus. Here, we showed for the first time that sub-lethal doses of atrazine impaired learning and memory in an invertebrate species.

Glyphosate also affected learning abilities of mosquito larvae even at the lowest dose: 100 □g/L. In addition, spray doses (5 mg/L) severely impaired individual learning and memory and increased locomotor activity. In acute toxicity tests, we also found a strong effect of glyphosate on memory 2 hours after training. These results confirm the previously demonstrated effects of glyphosate on insect cognition and add to the growing body of work showing negative effect of glyphosate on animals (Gill et al. 2018). Indeed, glyphosate reduced locomotor activity in nematodes, planarians, cockroaches and produced a slight and transient modification in bumblebees (García-Espiñeira 2018; Córdova López et al. 2019; Kanabar et al. 2021; Nouvian et al. 2023). Glyphosate has also been shown to alter spatial learning in honeybees, associative learning in honeybees and bumblebees and memory retention in honeybees (Herbert et al. 2014; Balbuena et al. 2015; Hernández et al. 2021; Nouvian et al. 2023). We hypothesise, in line with the literature, that the alteration primarily affects the central nervous system of mosquito larvae (Gill et al. 2018; Baglan et al. 2018).

Larvae reared at 10 mg/L of paracetamol slightly decreased their behavioural response during the training period, but we observed a clear difference between the 1st and the 10th trials for larvae reared at 1 mg/L and 100 mg/L. In addition, no effect of paracetamol was found for acute toxicity, and only an increase in the number of diving events was observed at the spray doses for the activity assessment. The literature assessing the effects of paracetamol on locomotor and cognitive abilities in animals remains scarce. We found two studies in mice and rats that observed an alteration of learning abilities in the presence of paracetamol. Regarding locomotor activity, Rakotondravelo et al. observed an increase in locomotion in rats, while two studies found a decrease in locomotion in zebrafish and mice (Viberg et al. 2014; Nogueira et al. 2019). Our study cannot determine the exact effects of paracetamol on invertebrate cognition, but we suggest that research into the potential effects and mechanisms of this

drug in the soil, water and organisms around human populations should continue (Hider-Mlynarz et al. 2018).

In our study, we combined the lowest concentrations of atrazine (200 µg/L) and paracetamol (1 mg/L). While these two concentrations alone had no effect, we found a significant increase in locomotion and an alteration in learning abilities in larvae reared with these two compounds in combination. Similarly, we conducted a series of experiments with the three compounds at field realistic concentrations measured directly in water. Alone, the pollutants revealed no effect on learning. However, when pollutants were presented in a mixture, learning was impaired while no effect on locomotion was found. It is important to assess the additive and possible synergistic effects of these compounds in mixtures. Indeed, aquatic organisms are constantly exposed to different types of pollutants and the possible synergistic effect of these pollutants would represent the worst case scenario for these organisms (Siviter et al. 2021). We chose to expose mosquito larvae to pure chemicals in order to access the effect of the combination without the influence of adjuvants, but it is important to point out that commercial herbicide formulation affect organisms not only by their main active ingredient but also by their overall formulation and residual products (Córdova López et al. 2019; Kanabar et al. 2021).

Taken together, these results support the global concern about the lack of knowledge about the impact of agrochemicals combined with pharmaceutical residues on aquatic ecosystems. Mosquitoes are known for their behavioural plasticity and their tolerance to pesticides (Poupardin et al. 2008). Their wide global distribution, tolerance to poor water conditions and their behaviour being affected by chemicals at low concentrations make them appropriate subjects to study the effects of pollutants on aquatic invertebrates. By altering the cognitive abilities of the larval stage, these pollutants increased energy expenditure by means of locomotor activity, which may affect their role as nutrient cycling or as food for predators, and affect the overall role of the food web dynamic in aquatic ecosystems (Kanabar et al. 2021). As mosquitoes are resistant to stressors, these toxicological effects are of concern for other species living in the same environments, and might be transferred by bioaccumulation to higher predators (Corbi et al. 2010).

In a previous paper, we presented an automated experimental approach for evaluating different parameters of the behaviour of mosquito larvae, notably, learning capacity and activity. Here we applied this concept to study the sublethal effects of water pollutants. Although our method is not yet an "ideal biomonitoring tool", as defined by some authors (Bonada et al. 2006), it revealed as an easy-to-use and modulable system for evaluating simultaneously 10 individuals, with high throughput data, making it appropriate for the assessment of risks associated with the presence of pollutants in the aquatic environment.

With this study, we tried to contribute to the field of cognitive ecotoxicology, using a model that is widely used and of great interest for human health. We believe that there is an urgent need to develop, communicate and standardise methods for measuring the impact of pollutants on these vulnerable ecosystems so that they can be used by policy makers to meet the next environmental, social and economic challenges.

Acknowledgements

Financial support for this work was granted by Region Centre-Val de Loire, France APR IR 2020 COMPORTATE. COMPORTATE is supported by Réseau MiDi - Milieux et Diversité, Pole DREAM - French Water Team, Zone Atelier de la Loire – LTER France Loire, Réseau ENTOMOCENTRE, Département d'Indre-et-Loire - Direction de l'Attractivité des Territoires (France).

We thank Elfie Perdereau for her help in handling the chemical products and the dilutions.

Author contributions

Martin Dessart: Conceptualization**,** Data curation, Formal analysis, Investigation, Methodology, Validation, Software, Visualization, Writing – original draft, Writing – review & editing. **Claudio R. Lazzari:** Conceptualization, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. **Fernando J. Guerrieri:**

Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

We declare we have no competing interests.

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Supplementary material

1. Data classification and filtering

Toxicity	Replicate	Treatment	Concentration		ID number		Detection rate	Vertical length (px)	Comment
Chronic - Residual doses	1	Atrazine	200 µg/L		10		0,99	389,5	
	2	Control n°1	/		9		0,89	392	ID#1 dead
	3	Atrazine Control n°1	200 µg/L	/	5	5	0,99	392,6	
	4	Control n°1 Atrazine	/	500 µg/L	5	5	0,98	390	
	5	Atrazine Control n°1	500 µg/L	/	5	5	0,99	391	
	6	Atrazine	500 µg/L		10		0,94	390,7	
	7	Atrazine	500 µg/L	200 µg/L	5	5	0,9	391,9	
	8	Control n°1 Atrazine	/	200 µg/L	5	5	0,95	395,6	
	9	Atrazine Control n°1	200 µg/L	/	4	5	0,96	395,7	ID#2 dead
	10	Control n°1 Atrazine	/	500 µg/L	5	5	0,98	394,1	
	11	Glyphosate	200 µg/L		10		0,93	389,9	
	12	Glyphosate Control n°2	200 µg/L	/	5	5	0,92	393,2	
	13	Glyphosate	100 µg/L		10		0,85	393,5	
	14	Glyphosate Control n°2	100 µg/L	/	5	5	0,91	395,1	
	15	Control n°2 Glyphosate	/	100 µg/L	5	5	0,99	394,8	
	16	Glyphosate Control n°2	100 µg/L	/	5	5	0,89	395,6	
	17	Paracetamol Control n°2	1 mg/L	/	5	5	0,93	393	
	18	Control n°2 Paracetamol	/	1 mg/L	4	6	0,93	389,7	
	19	Paracetamol	10 mg/L	1 mg/L	5	5	0,97	393,5	
	20	Paracetamol	1 mg/L	10 mg/L	5	5	0,98	389,2	
	21	Paracetamol Glyphosate	1 mg/L	200 µg/L	5	5	0,94	396,7	
	22	Glyphosate Paracetamol	200 µg/L	1 mg/L	5	5	0,99	394	
	23	Control n°2	/		10		0,96	390	
	24	Paracetamol	10 mg/L		10		0,96	395,1	
	25	Paracetamol	10 mg/L		10		0,98	391,1	
	26	Glyphosate	100 µg/L	200 µg/L	5	5	0,87	394,2	
Chronic - Spray doses	27	Control n°3	/		10		0,98	309,6	
	28	Control n°3	/		9		0,99	391,7	ID#2 dead
	29	Control n°3	/		2		0,97	389,6	ID#1 Mix2, ID#2 to ID#3 Control, ID#4 dead, ID#5 to ID#10 Mix3
	30	Control n°3	/		10		0,99	389,6	
	31	Atrazine	2 mg/L		10		0,96	390,3	
	32	Atrazine	2 mg/L		10		0,98	390,9	
	33	Atrazine	2 mg/L		10		0,99	391	
	34	Control n°3	/		10		0,99	393,4	
	35	Glyphosate	2 mg/L		10		0,95	391,3	
	36	Glyphosate	2 mg/L		10		0,95	390,8	
	37	Glyphosate	2 mg/L		10		0,91	391,7	
	38	Paracetamol	100 mg/L		10		0,94	384,8	
	39	Paracetamol	100 mg/L		10		0,95	390,3	
	40	Control n°4	/		9		0,96	400,9	ID#8 did not respond to vibration
	41	Control n°4	/		10		0,99	400,4	
	42	Control n°4	/		7		0,95	399,3	ID#2, ID#3 did not respond to vibration, ID#6 did not respond to vibration
	43	Paracetamol	100 mg/L		10		0,88	392,7	

Chronic - Field doses	44	Control n°5	/	10	0.99	395.2	
	45	Control n°5	/	10	0.99	395.2	
	46	Paracetamol	6 µg/L	10	0.89	399.8	
	47	Glyphosate	0.126 µg/L	10	0.94	402	
	48	Control n°5	/	10	0.99	391.3	
	49	Atrazine	2.3 µg/L	9	0.9	399.8	ID#5 did not respond to vibration
	50	Paracetamol	6 µg/L	10	0.98	397	
	51	Glyphosate	0.126 µg/L	10	0.91	398.5	
	52	Atrazine	2.3 µg/L	9	0.95	399.2	ID#10 did not respond to vibration
	53	Paracetamol	6 µg/L	10	0.99	403.9	
	54	Glyphosate	0.126 µg/L	10	0.99	399.2	
	55	Atrazine	2.3 µg/L	9	0.9	400.7	ID#2 did not respond to vibration
	56	Mixture N°1	G: 0.126 µg/L | A:2.3 µg/L | P: 6 µg/L	10	0.98	390.1	
	57	Control n°6	/	10	0.98	309.6	
	58	Mixture N°1	G: 0.126 µg/L | A:2.3 µg/L | P: 6 µg/L	5	0.96	387.5	ID#6 to ID#10 from Glyphosate-only group
	59	Control n°6	/	9	0.99	391.7	ID#2 dead
	60	Mixture N°1	G: 0.126 µg/L | A:2.3 µg/L | P: 6 µg/L	9	0.93	388.4	ID#10 transformed in pupae
	61	Mixture N°2	P:1 mg/L | A:200 µg/L	10	0.99	390.3	
	62	Mixture N°2	P:1 mg/L | A:200 µg/L	10	0.98	394.4	
	63	Mixture N°2 Control n°6	P:1 mg/L | A:200 µg/L /	7 2	0.97	389.6	ID#1 Mix2, ID#2 to ID#3 Control, ID#4 dead, ID#5 to ID#10 Mix3
	64	Control n°6	/	10	0.99	389.6	
	65	Control n°6	/	10	0.99	393.4	
Acute - Spray doses	66	Control n°7	/	9	0.97	402.3	ID#6 dead
	67	Control n°7	/	10	0.9	400.9	
	68	Control n°7	/	10	0.98	405.7	
	69	Glyphosate	2 mg/L	10	0.92	405.8	
	70	Atrazine	2 mg/L	9	0.88	403.3	ID#10 not tracked
	71	Paracetamol	100 mg/L	10	0.94	404.7	
	72	Atrazine	2 mg/L	10	0.96	404.1	
	73	Atrazine	2 mg/L	8	0.93	404.4	ID#5, ID#8 did not respond to vibration
	74	Glyphosate	2 mg/L	10	0.89	406.3	
	75	Paracetamol	100 mg/L	10	0.94	405.6	
	76	Paracetamol	100 mg/L	10	0.96	403.9	
	77	Glyphosate	2 mg/L	10	0.97	405.9	

Supplementary Table T1: Details for each experiment performed. Toxicity represents the type of toxicity studied. Each replicate represents 10 individuals trained during one session. Treatment represents the rearing (for chronic toxicity) or training (for acute toxicity) water treatment. If the cell is divided in two, it means that we tested 5 individuals for one treatment and 5 for another treatment. The concentration column refers to the concentration of chemicals used and ID number correspond to the number of individual for each replicate. Detection rate was calculated as the ratio between the maximum frame number and the actual frame number identified by the tracking software. Vertical length was calculated as the difference between the maximum and the minimum individual position measured on each video by the tracking software.

Chronic pollution

	Field doses								*Spray doses*					
	/	200 µg/L	500 µg/L	/	100 µg/L	200 µg/L	1 mg/L	10 mg/L	/	2 mg/L	/	2 mg/L	100 mg/L	
	Control 1	Atrazine	Atrazine	Control 2	Glyphosate	Glyphosate	Paracetamol	Paracetamol	Control 3	Atrazine	Control 4	Glyphosate	Paracetamol	
Individuals trained	34	**30**	29	44	**30**	**30**	31	30	41	30	26	30	30	
Trials per individuals	340	300	290	440	300	300	310	300	410	300	260	300	300	
Trials filtered by position	258	234	225	367	255	216	241	241	339	248	223	248	240	
% Trials removed	24.1%	22.0%	22.4%	16.6%	15.0%	28.0%	22.3%	19.7%	17.3%	17.3%	14.2%	17.3%	20.0%	
Trials filtered by going up	257	234	224	362	254	214	240	240	337	248	222	244	240	
% Trials removed	0.4%	0.0%	0.4%	1.4%	0.4%	0.9%	0.4%	0.4%	0.6%	0.0%	0.4%	1.6%	0.0%	
Total % Trials removed	24.4%	22.0%	22.8%	17.7%	15.3%	28.7%	22.6%	20.0%	17.8%	17.3%	14.6%	18.7%	20.0%	

Chronic pollution **Acute pollution**

	Realistic doses							*Spray doses*				
	/	2.3 µg/L	0.126 µg/L	6 µg/L	/	A:2.3 µg/L G: 0.126 µg/L P: 6 µg/L	A:200 µg/L P:1 mg/L	/	2 mg/L	2 mg/L	100 mg/L	
	Control 5	Atrazine	Glyphosate	Paracetamol	Control 6	Mix N°1	Mix N°2	Control 7	Atrazine	Glyphosate	Paracetamol	Total
Individuals trained	30	27	30	30	41	25	26	29	27	30	30	740
Trials per individuals	300	270	300	300	410	250	260	319	297	330	330	7516
Trials filtered by position	237	209	228	215	342	203	192	256	217	269	279	5982
% Trials removed	21.0%	22.6%	24.0%	28.3%	16.6%	18.8%	26.2%	19.7%	26.9%	18.5%	15.5%	**20.4%**
Trials filtered by going up	234	205	228	214	340	203	190	255	212	269	279	5945
% Trials removed	1.3%	1.9%	0.0%	0.5%	0.6%	0.0%	1.0%	0.4%	2.3%	0.0%	0.0%	0.6%
Total % Trials removed	22.0%	24.1%	24.0%	28.7%	17.1%	18.8%	26.9%	20.1%	28.6%	18.5%	15.5%	**20.9%**

Supplementary Table T2: Summary of the filtering steps. For each species, 25 to 59 individuals were trained. When the individual's position was close to the bottom, the response to the trial was removed, accounting for a total of 20.5% of trials removed. The trajectory of an individual moving upwards during a stimulation was rare, representing only 0.6% of trials.

Treatment	Control 1	Atrazine	Atrazine	Control 2	Glyphosate	Glyphosate	Paracetamol	Paracetamol	Control 3	Atrazine	Control 4	Glyphosate	Paracetamol
Concentration	/	200 µg/L	500 µg/L	/	100 µg/L	200 µg/L	1 mg/L	10 mg/L	/	2 mg/L	/	2 mg/L	100 mg/L
χ 2	5.313	13.697	11.879	11.231	13.565	7.023	6.286	8	3.028	8	10.421	11.5	8.333
df	9	9	9	9	9	9	9	9	9	9	9	9	9
P =	0.81	0.13	0.22	0.26	0.14	0.63	0.71	0.53	0.96	0.53	0.32	0.24	0.5

Treatment	Control 5	Atrazine	Glyphosate	Paracetamol	Control 6	Mix	Mix	Control 7	Atrazine	Glyphosate	Paracetamol
Concentration	/	2.3 µg/L	0.126 µg/L	6 µg/L	/	G: 0.126 µg/L + A:2.3 µg/L + 6 µg/L	P:1 mg/L + A:200 µg/L	/	2 mg/L	2 mg/L	100 mg/L
χ 2	4.909	9.615	12.421	8.88	4.286	11.936	8.571	6.812	8.306	7.37	10.471
df	9	9	9	9	9	9	9	10	10	10	10
P =	0.84	0.38	0.19	0.45	0.89	0.22	0.48	0.74	0.6	0.67	0.4

Supplementary Table T3: Similar to Dessart et al. (2023, 2024), we tested whether the number of trials deleted by the criterion depended on the trial number, by applying a chi-squared test to the deleted trials as a function of the trial number for each treatment. For all treatments, the deleted trials were not specific to any trial number.

2. Spontaneous activity

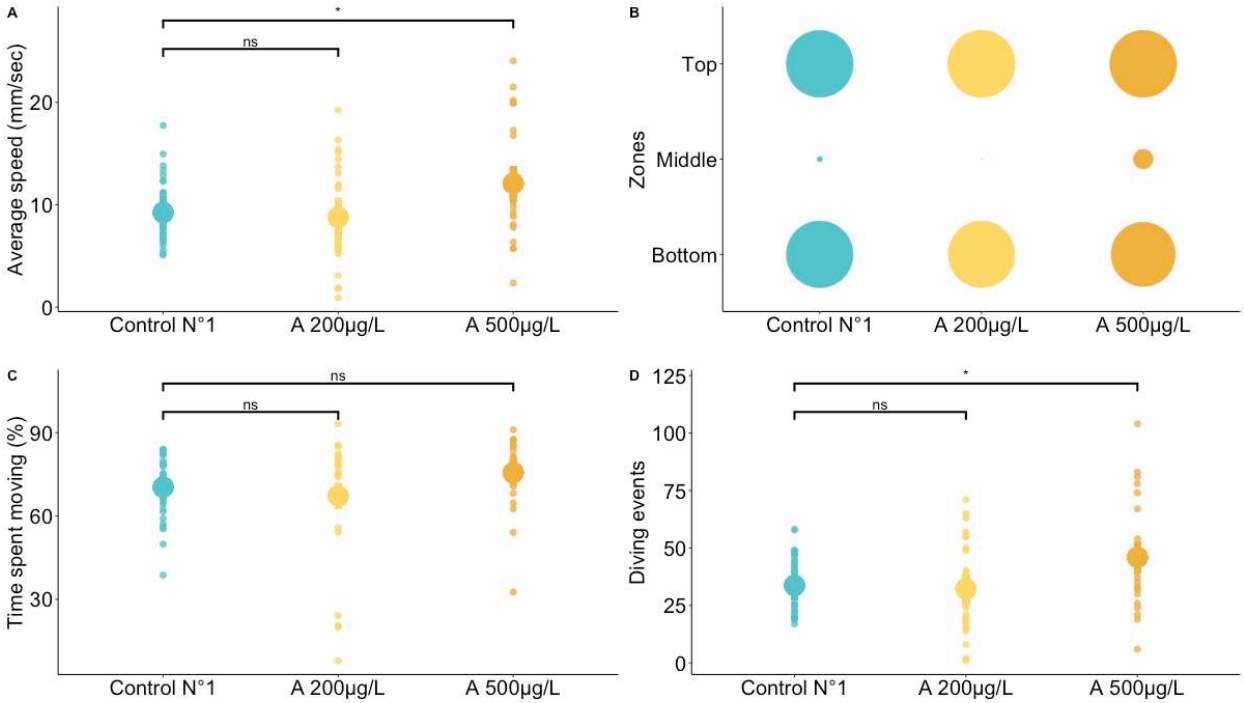
		Treatment	Atrazine	Atrazine	Glyphosate	Glyphosate	Paracetamol	Paracetamol	Atrazine	Glyphosate	Paracetamol
		Concentration	200 µg/L	500 µg/L	100 µg/L	200 µg/L	1 mg/L	10 mg/L	2 mg/L	2 mg/L	100 mg/L
Activity during familiarisation	Average speed	t-ratio	**-2.427**	**-3.593**	-0.165	-0.206	1.524	1.337	1.371	-1.365	-0.351
		df	**85**	**85**	85	85	85	85	50	83	83
		P	**0.05**	**< 0.01**	0.99	0.98	0.28	0.38	0.18	0.36	0.93
	Time spent moving	t-ratio	-0.75	-2.312	-0.709	-0.318	0.94	0.532	1.545	-1.321	0.757
		df	85	85	85	85	85	85	50	83	83
		P	0.73	0.06	0.76	0.95	0.62	0.86	0.13	0.39	0.73
	Diving events	t-ratio	**-1.99**	**-3.565**	-0.217	1.2	0.659	-0.313	1.168	-1.549	**-2.906**
		df	**85**	**85**	85	85	85	85	50	83	**83**
		P	**0.05**	**< 0.01**	0.98	0.46	0.79	0.95	0.25	0.27	**0.01**
Activity during inter-trial intervals	Average speed	t-ratio	0.441	**2.603**	-0.591	0.997	1.043	0.954	1.438	**-3.033**	-2.062
		df	90	**90**	101	101	102	102	69	**83**	83
		P	0.9	**0.03**	0.83	0.58	0.55	0.61	0.15	**< 0.01**	0.1
	Time spent moving	t-ratio	0.832	-1.362	-1.117	-0.237	1.043	0.954	1.357	-1.589	0.219
		df	90	90	101	101	102	102	69	83	83
		P	0.68	0.37	0.51	0.97	0.55	0.61	0.18	0.26	0.97
	Diving events	t-ratio	0.341	**-2.755**	-1.017	1.079	0.958	-0.016	0.237	**-4.622**	**-4.344**
		df	90	**90**	101	101	102	102	69	**83**	**83**
		P	0.94	**0.01**	0.57	0.53	0.61	0.99	0.81	**< 0.0001**	**< 0.001**

		Treatment	Atrazine	Glyphosate	Paracetamol	Mix	Mix	Atrazine	Glyphosate	Paracetamol
		Concentration	2.3 µg/L	0.126 µg/L	6 µg/L	G: 0.126 µg/L + A:2.3 µg/L + 6 µg/L	P:1 mg/L + A:200 µg/L	2 mg/L	2 mg/L	100 mg/L
Activity during familiarisation	Average speed	t-ratio	-1.153	-0.822	0.459	0.037	**-1.988**	1.858	-0.312	1.45
		df	113	113	113	89	**89**	114	114	114
		P	0.66	0.84	0.97	0.99	**0.05**	0.25	0.99	0.47
	Time spent moving	t-ratio	-0.478	-1.48	1.125	-0.67	-0.812	1.623	2.098	**3.008**
		df	113	113	113	89	89	114	114	**114**
		P	0.96	0.45	0.67	0.78	0.7	0.37	0.16	**0.02**
	Diving events	t-ratio	-1.115	-0.776	0.111	-0.346	**-3.64**	1.502	0.606	1.056
		df	113	113	113	89	**89**	114	114	114
		P	0.68	0.87	0.99	0.94	**< 0.01**	0.44	0.93	0.72
Activity during inter-trial intervals	Average speed	t-ratio	-2.547	-0.707	1.166	-0.69	**-3.053**	2.194	2.432	2.185
		df	113	113	113	89	**89**	112	112	112
		P	0.06	0.89	0.65	0.77	**< 0.01**	0.13	0.08	0.13
	Time spent moving	t-ratio	-1.279	-1.032	1.533	-0.52	-2.033	1.716	1.934	**2.76**
		df	113	113	113	89	89	112	112	**112**
		P	0.57	0.73	0.42	0.86	0.11	0.32	0.22	**0.03**
	Diving events	t-ratio	-0.22	1.896	**-2.789**	-0.042	**-3.602**	0.279	1.465	0.526
		df	113	113	**113**	89	**89**	112	112	112
		P	0.99	0.24	**0.03**	0.99	**< 0.01**	0.9	0.46	0.95

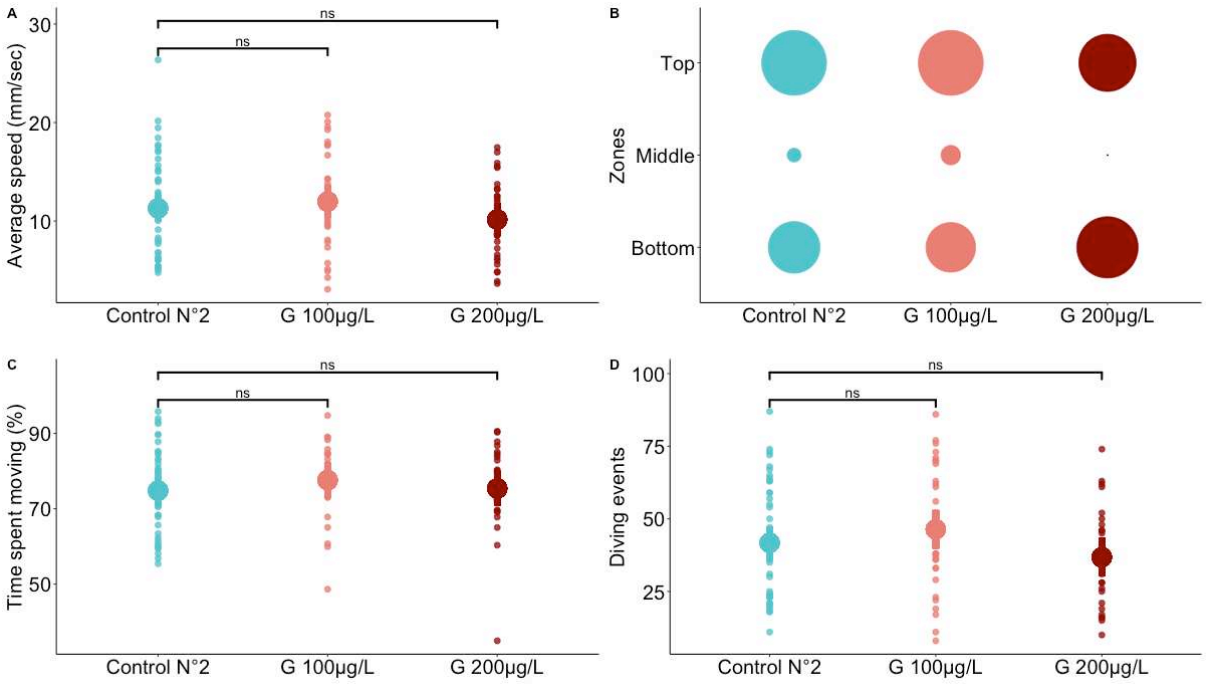
Supplementary Table T4: shows all the comparison between treatment and control.

3. Spontaneous activity during inter-trial intervals

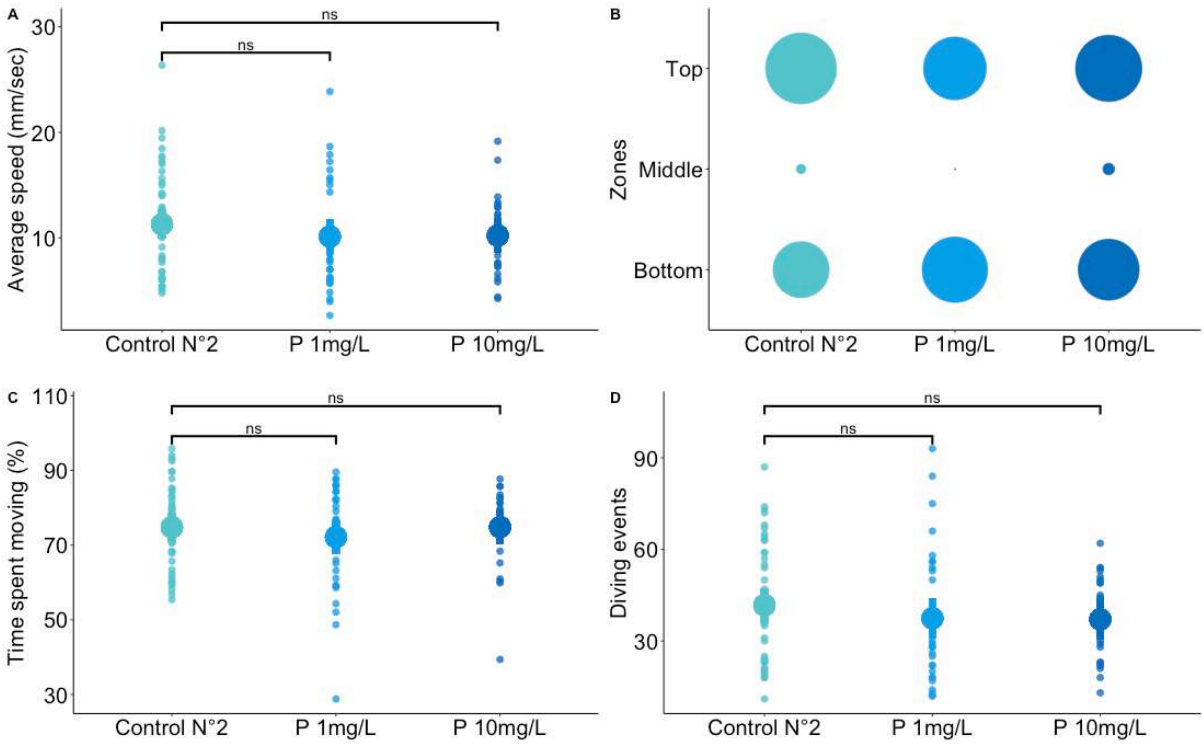
Here are the graphs representing differences during the **inter-trial intervals**.



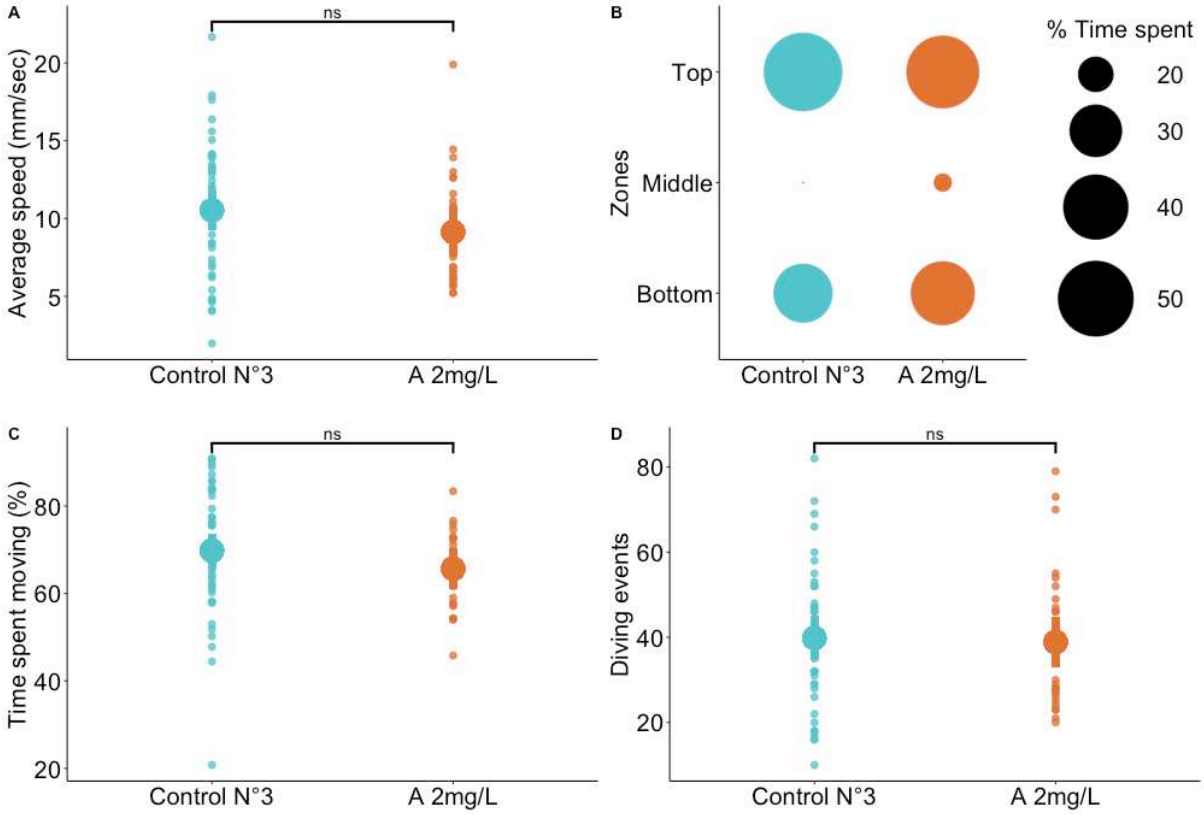
Supplementary Figure S1: Spontaneous locomotor activity for larvae reared in atrazine at field doses during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant; *P<0.05.



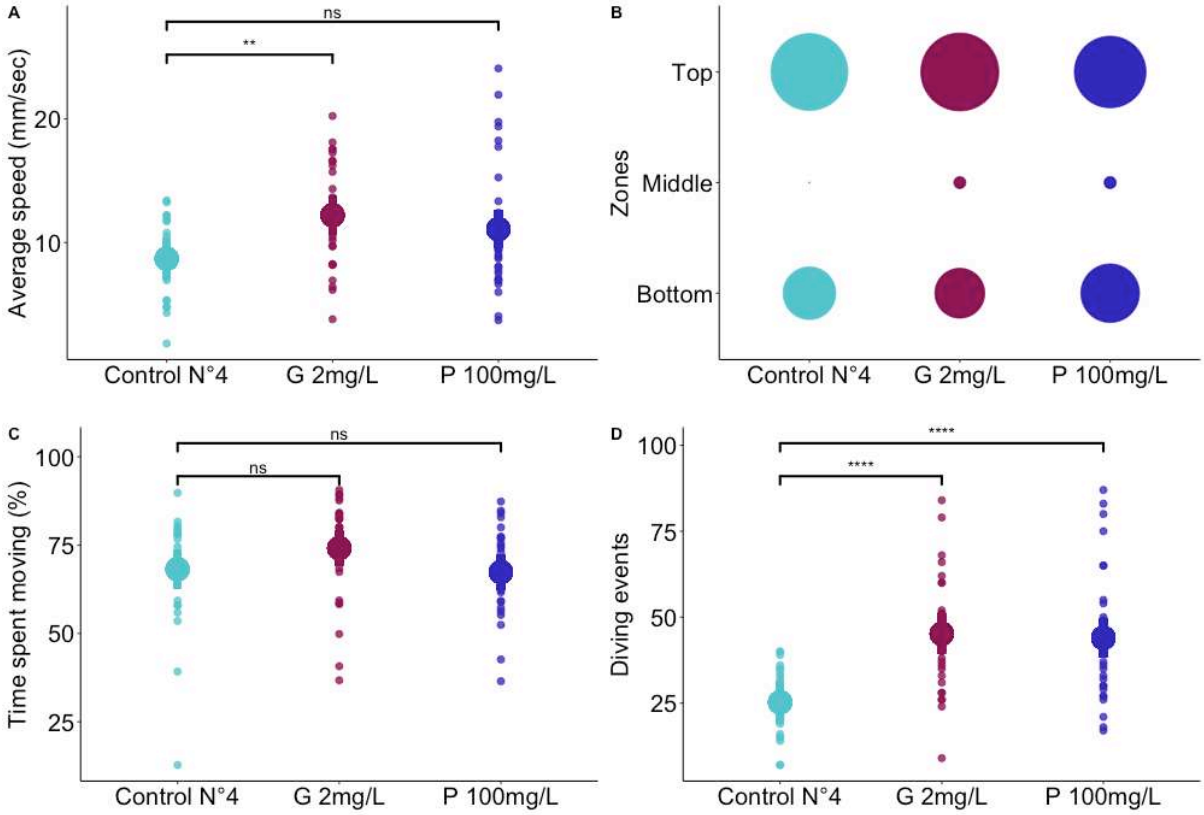
Supplementary Figure S2: Spontaneous locomotor activity for larvae reared in glyphosate at field doses during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant.



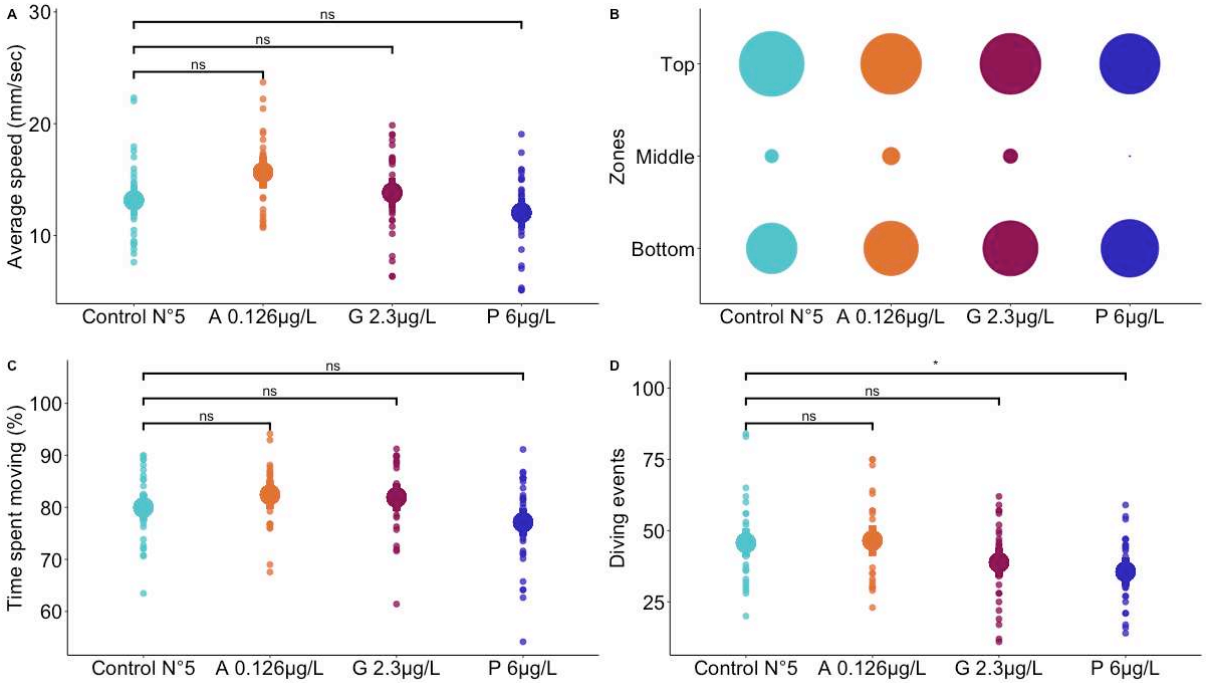
Supplementary Figure S3: Spontaneous locomotor activity for larvae reared in paracetamol at field doses during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant.



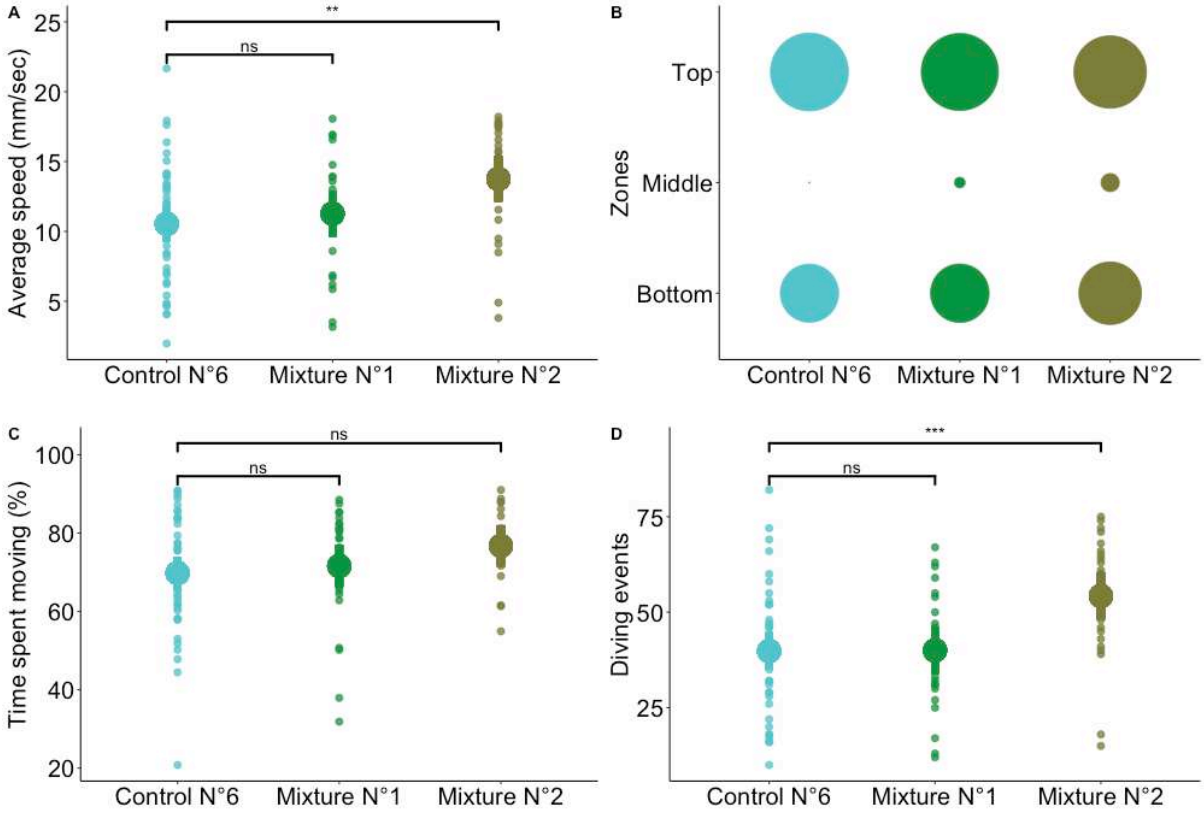
Supplementary Figure S4: Spontaneous locomotor activity for larvae reared in atrazine at spray doses during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. A= Atrazine. NS, not significant.



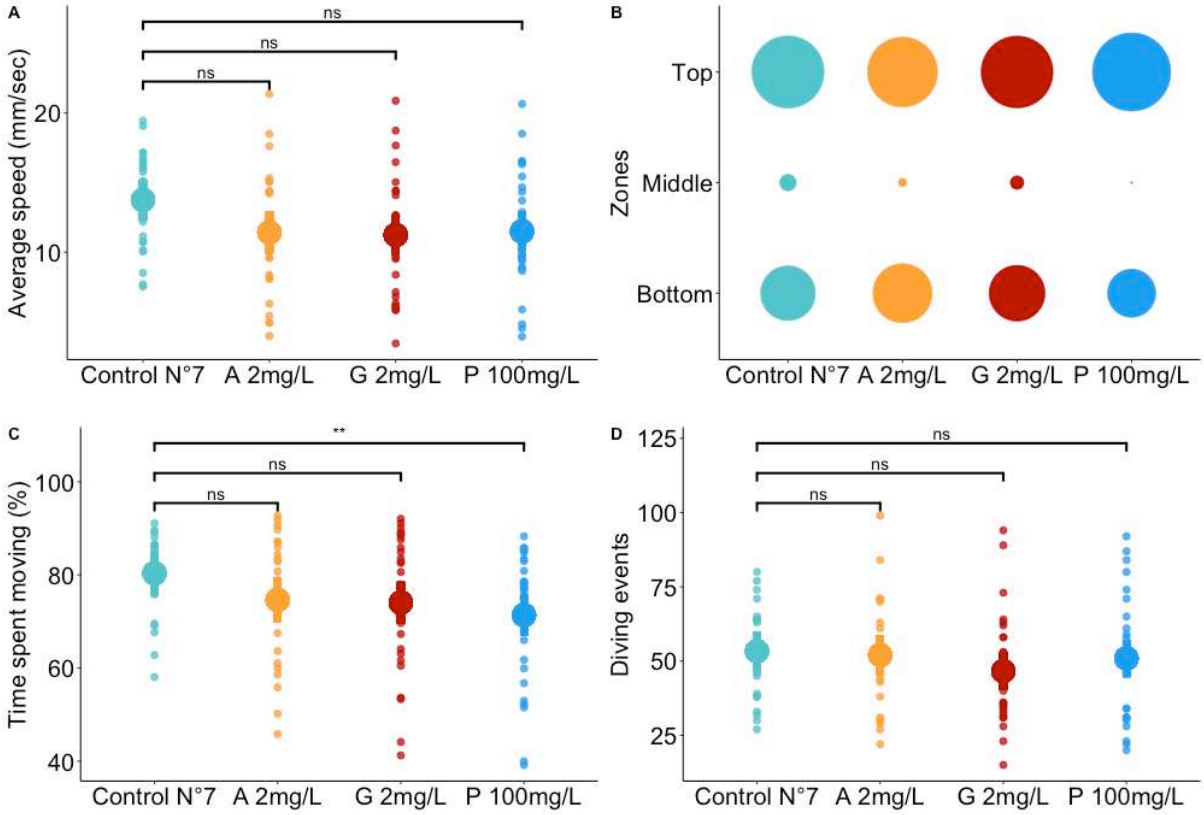
Supplementary Figure S5: Spontaneous locomotor activity for larvae reared in glyphosate and paracetamol at spray doses during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. G = Glyphosate, P = Paracetamol. NS, not significant; **P<0.01, ****P<0.0001.



Supplementary Figure S6: Spontaneous locomotor activity for larvae reared alone at field realistic doses during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. A= Atrazine, G = Glyphosate, P = Paracetamol. NS, not significant; *P<0.05.



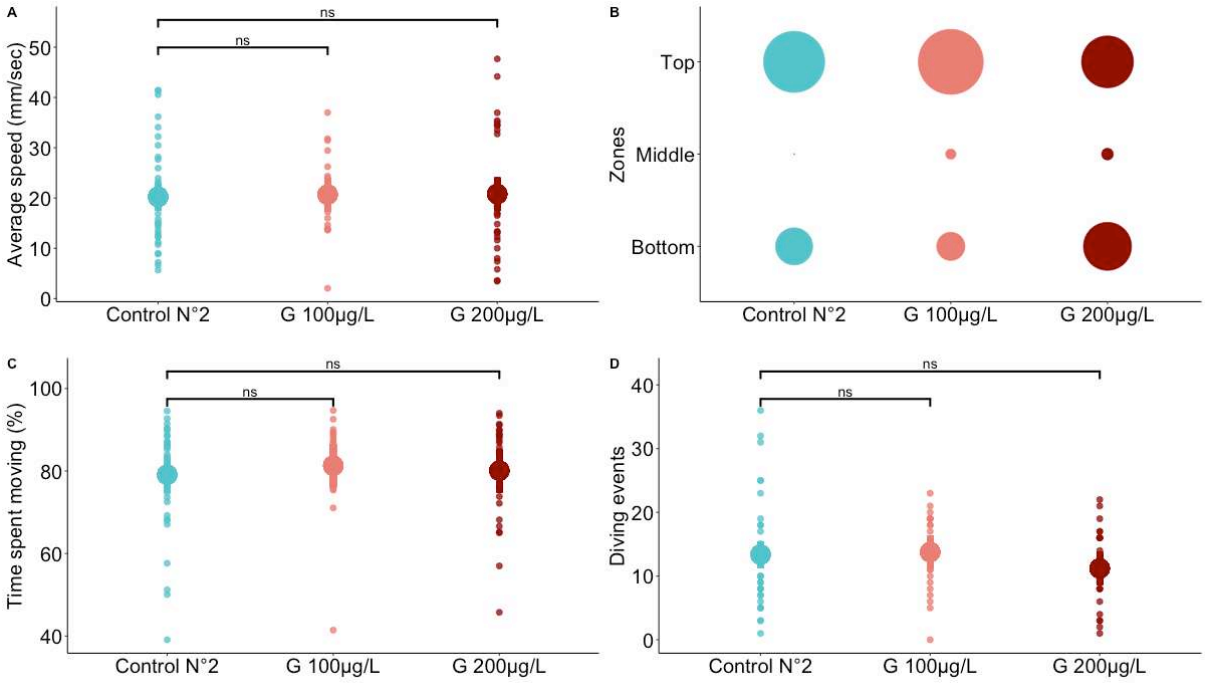
Supplementary Figure S7: Spontaneous locomotor activity for larvae reared in mixture at field realistic doses during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant; **P<0.01, ***P<0.001.



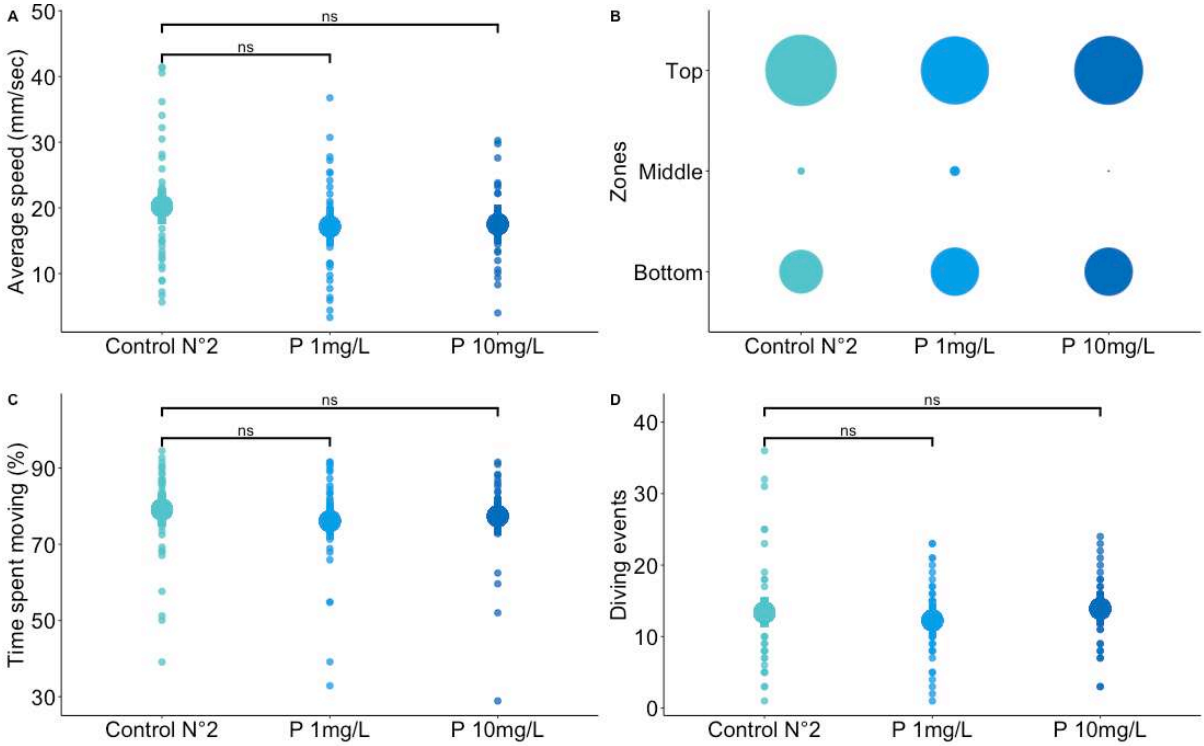
Supplementary Figure S8: Spontaneous locomotor activity for larvae reared at spray doses for acute toxicity during inter-trials intervals. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant; **P<0.01.

4. Spontaneous activity during the familiarisation period

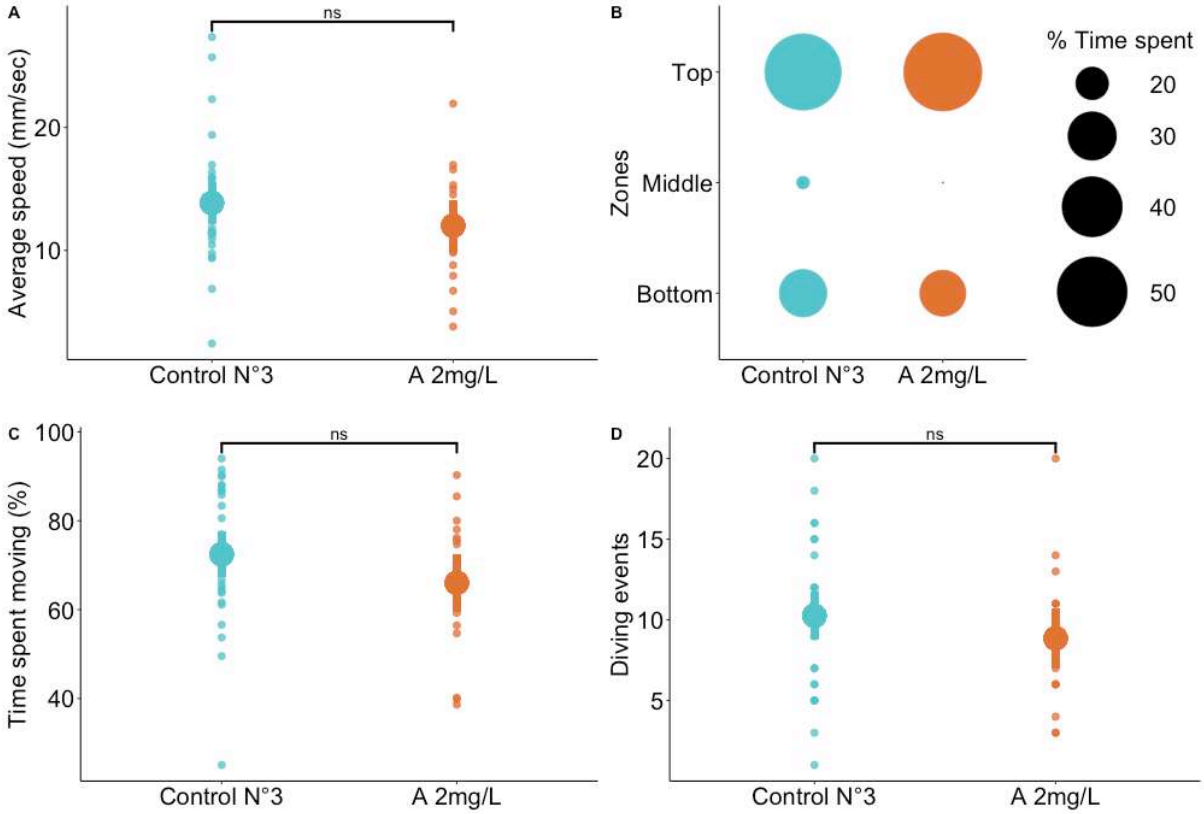
Here are the graphs representing differences during the **familiarisation period**.



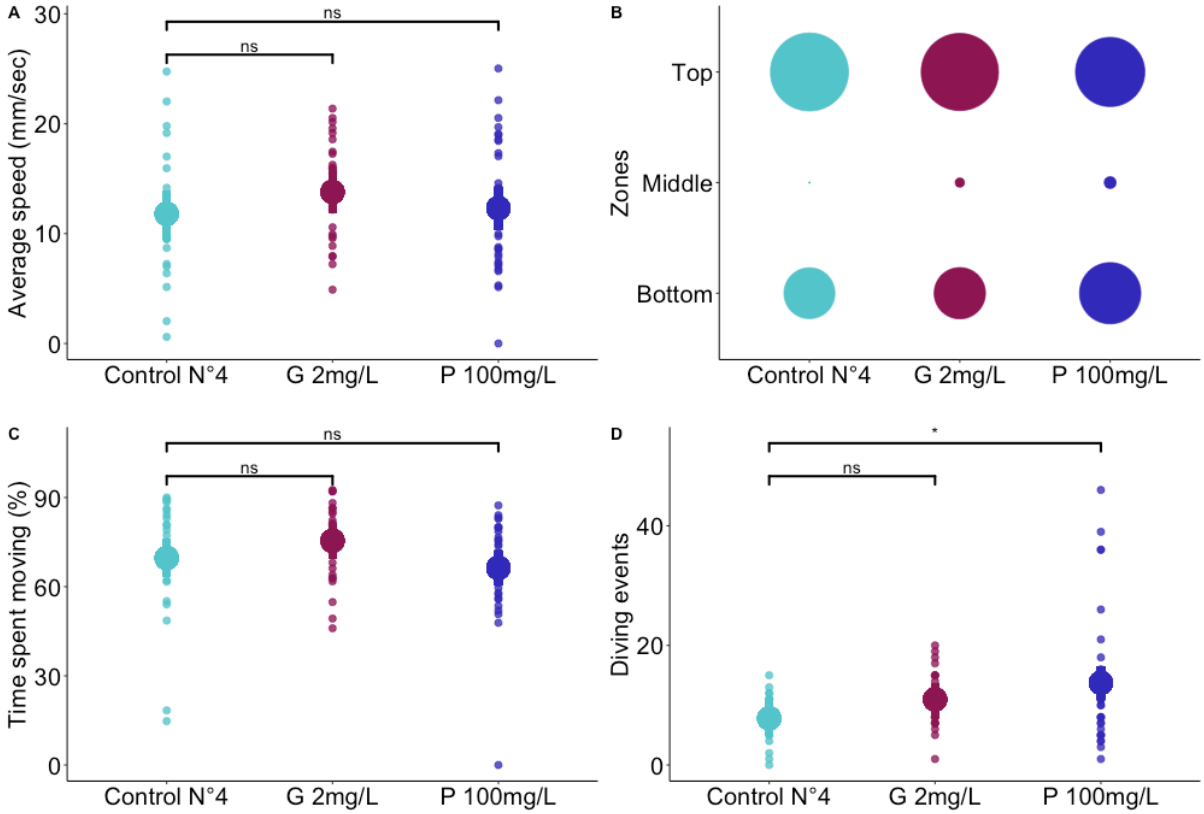
Supplementary Figure S9: Spontaneous locomotor activity for larvae reared in glyphosate at field doses during **familiarisation**. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant.



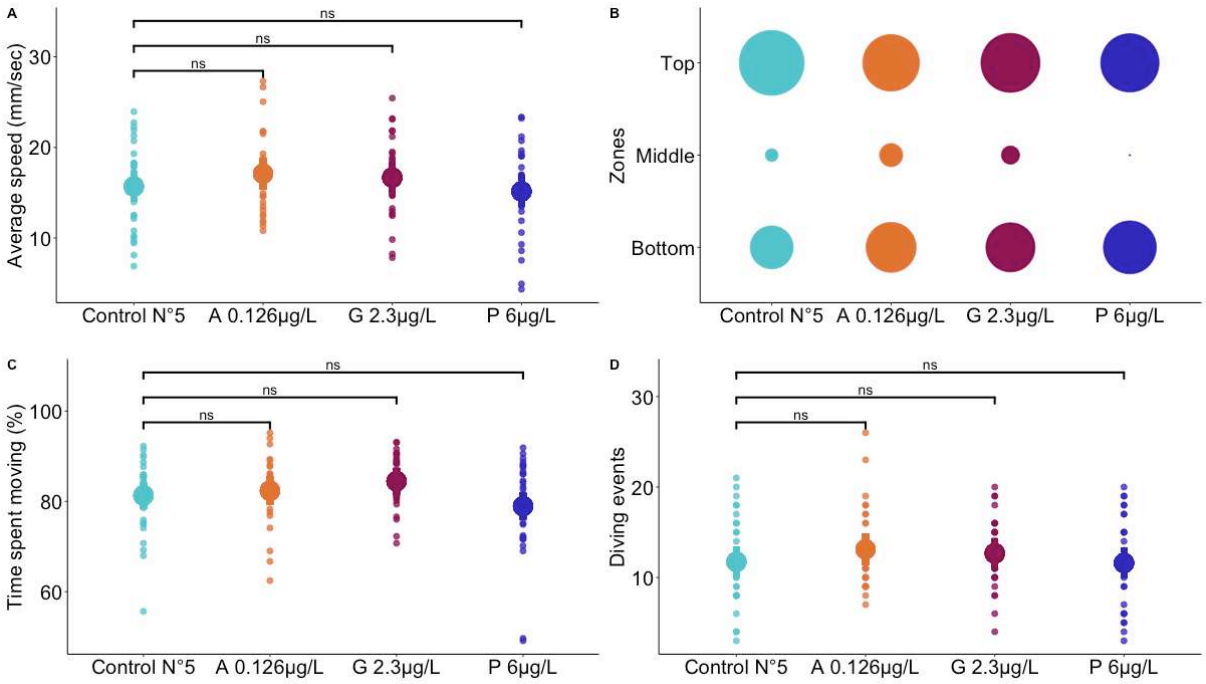
Supplementary Figure S10: Spontaneous locomotor activity for larvae reared in paracetamol at field doses during **familiarisation**. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant.



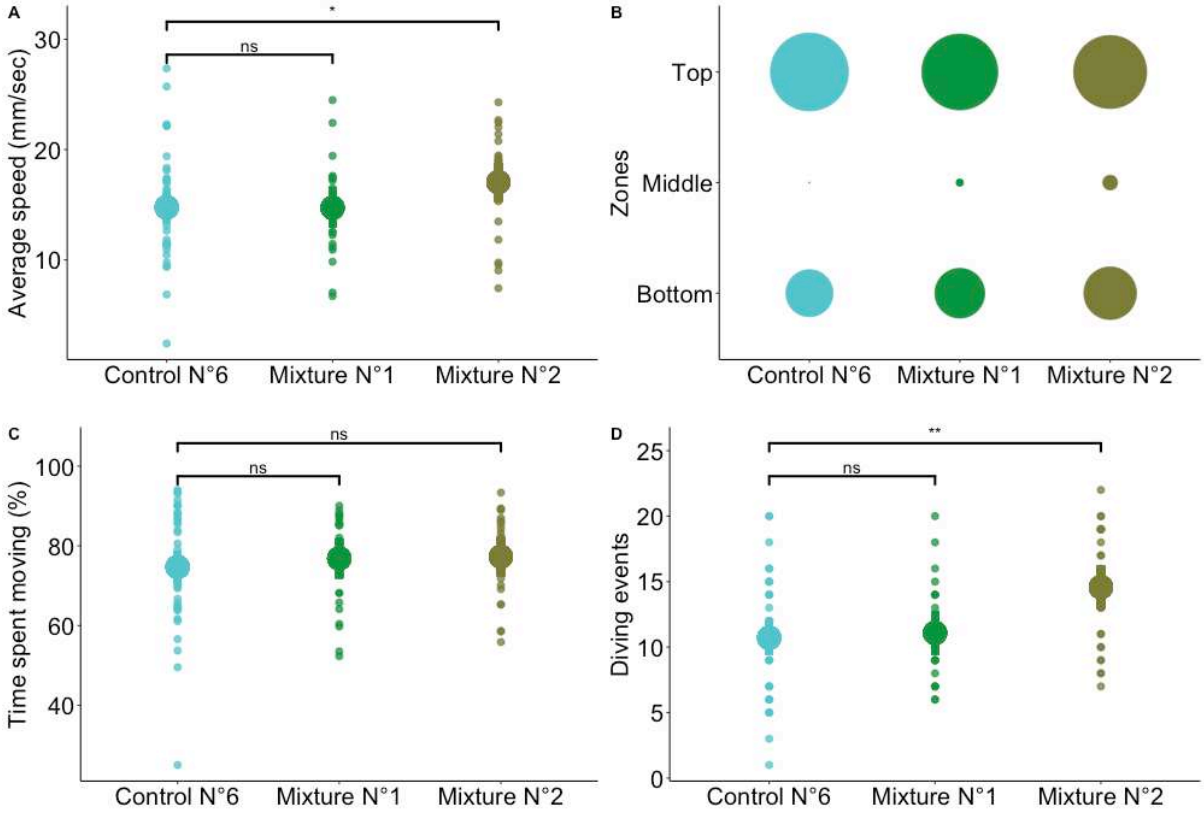
Supplementary Figure S11: Spontaneous locomotor activity for larvae reared in atrazine at spray doses during **familiarisation**. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. A= Atrazine. NS, not significant.



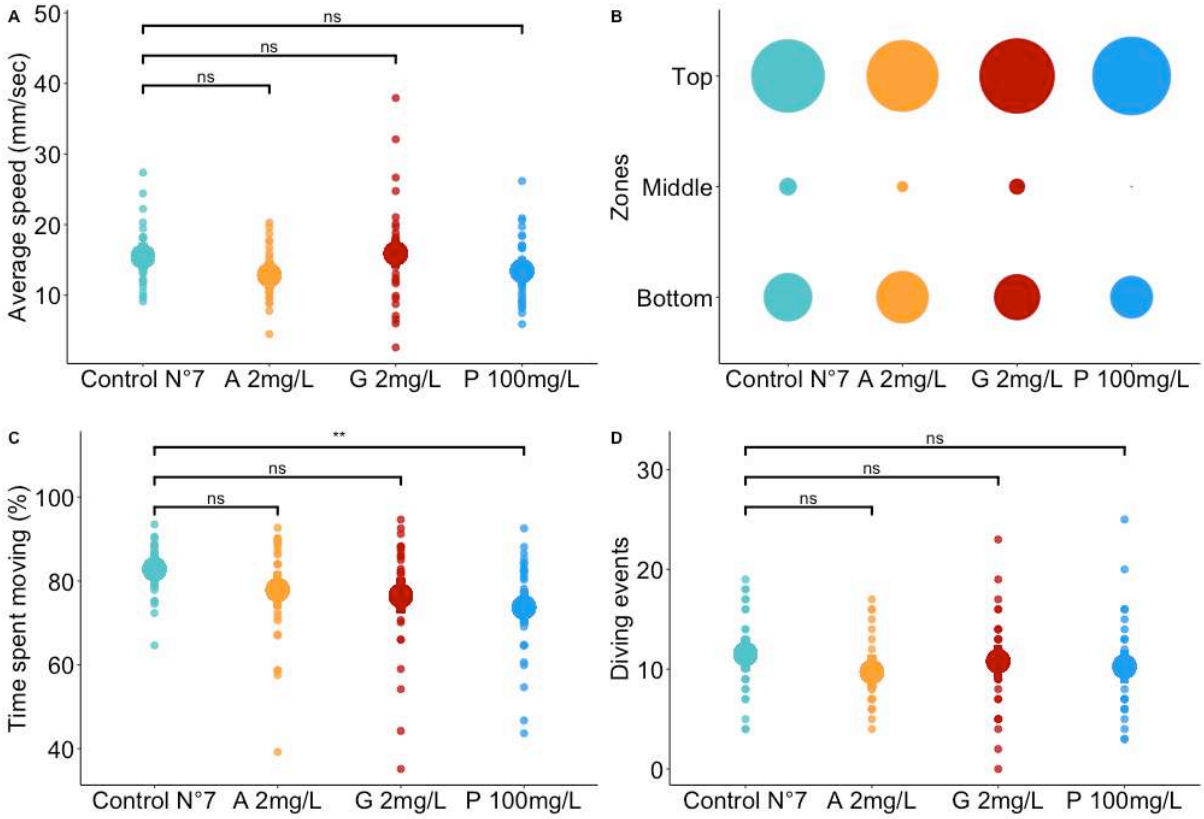
Supplementary Figure S12: Spontaneous locomotor activity for larvae reared in glyphosate and paracetamol at spray doses during **familiarisation**. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. G = Glyphosate, P = Paracetamol. NS, not significant; *P<0.05.



Supplementary Figure S13: Spontaneous locomotor activity for larvae reared alone at field realistic doses during **familiarisation**. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. A= Atrazine, G = Glyphosate, P = Paracetamol. NS, not significant.



Supplementary Figure S14: Spontaneous locomotor activity for larvae reared in mixture at field realistic doses during **familiarisation**. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant; *P<0.05, **P<0.01.

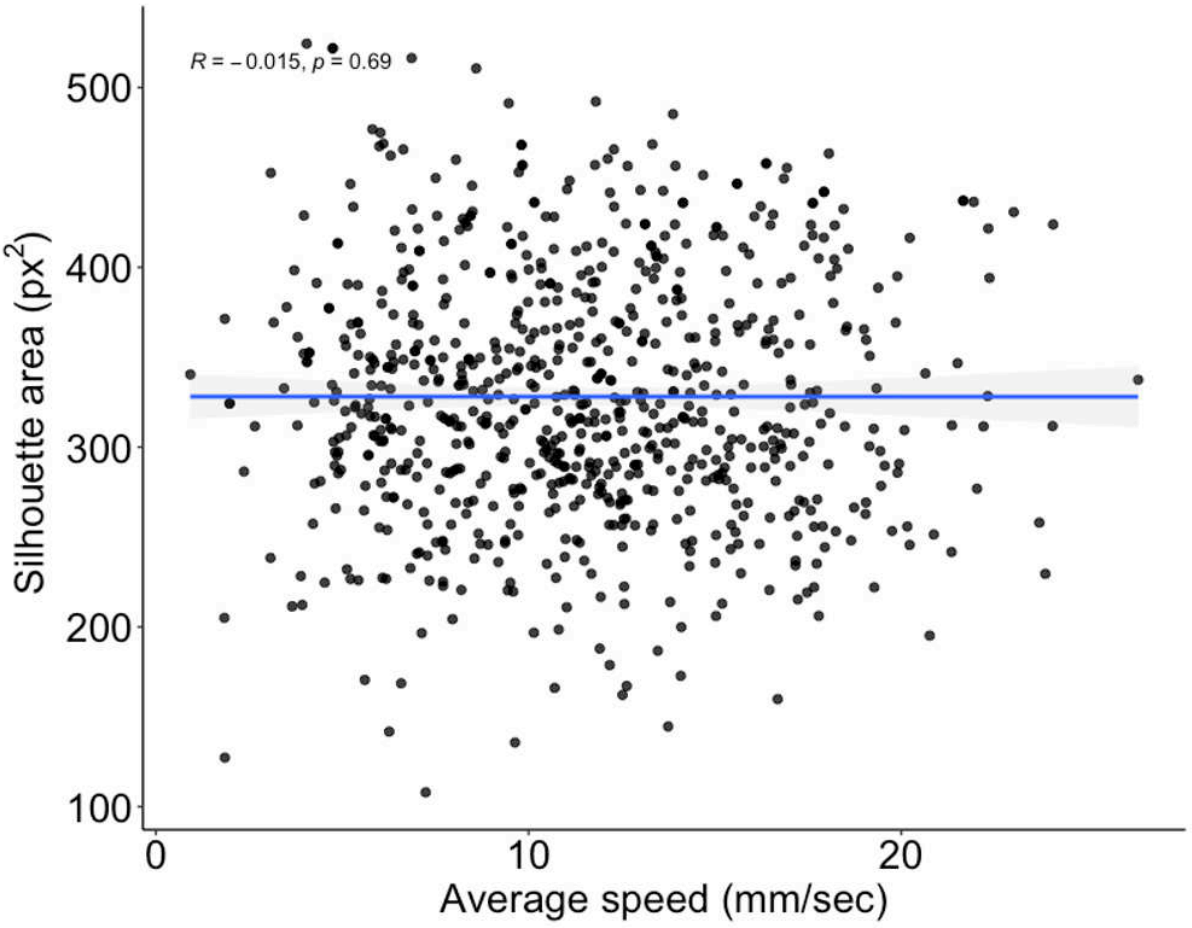


Supplementary Figure S15: Spontaneous locomotor activity for larvae reared at spray doses for acute toxicity during **familiarisation**. A) Average speed (mm/sec) for each individual during the training period. B) Average time spend in each zone (%). C) Average time spend moving (TM, %). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Dark square indicates mean value for one treatment. NS, not significant; **P<0.01.

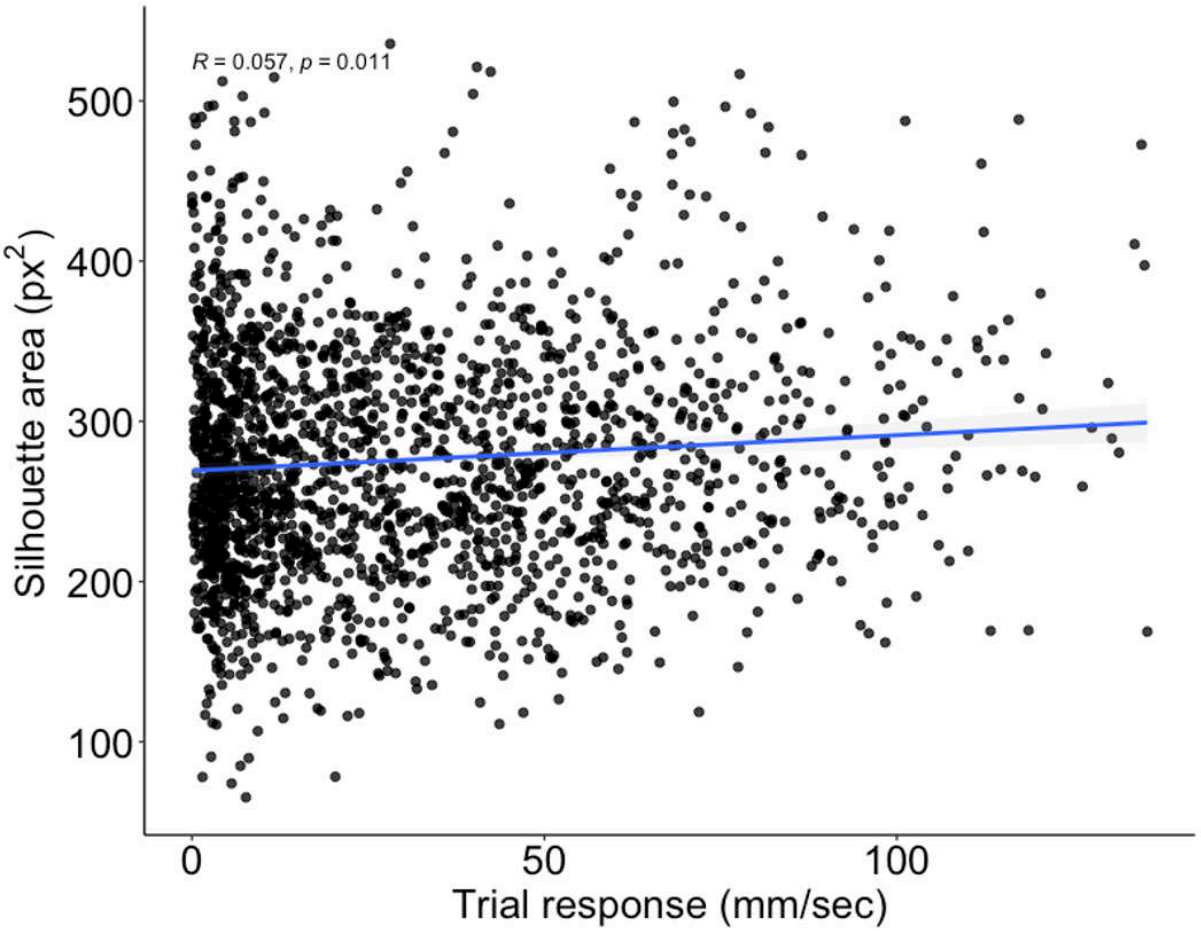
5. Correlations between spontaneous activity and silhouette area

We used the contour of the individual over the videos to check the correlation between the individual surface detected by the tracking software and the response at the first stimulation and spontaneous activity.

We compared the average silhouette area of all individuals retained for this study (n = 740) to their response at the first trial during training (Figure 14) and to their average speed during the training period (Figure 13). While differences in silhouette area could be observed, there were no correlations between the response at the first trial or the average speed, and the silhouette area (R = 0.057, P = 0.011; R = -0.015; P = 0.69, Figure S9 and S10 below).



Supplementary Figure S16: Average silhouette area correlated with individual average speed. Points indicate the mean value for an individual. Blue line corresponds to linear correlation. Grey shades indicate 95% confidence interval for the average speed.



Supplementary Figure S17: Average silhouette area correlated with individual response at the 1st trial. Points indicate the mean value for an individual. Blue line corresponds to linear correlation. Grey shades indicate 95% confidence interval for the average speed.

Chapitre 5 : Les conditions environnementales affectent l'activité et la cognition des larves de moustique, mais pas leur sensibilité à un stimulus visuel

if biodiversity really matters for the planet, and is essential for humanity's well-being, we need to get real about what it will take to conserve it for future generations […] But if not us, who?

Aaron M. **Ellison** (2016) It's time to get real about conservation. Nature

Ce chapitre a fait l'objet d'un manuscrit qui est au format **préliminaire**. Il s'inscrit au sein du **projet régional COMPORTATE**, financé par la région Centre-Val de Loire, qui a pour but d'évaluer les écosystèmes de la région par une vision pluridisciplinaire. Trois autres approches que celle présentée dans ce travail de thèse sont à l'appui : **mesures physico-chimiques** des caractéristiques organiques l'eau ; **détection de polluants** par mesures de chimie analytique ; observation de la **biodiversité des odonates**. Ce projet a ainsi démarré en 2021 une campagne de terrain de trois ans sur plusieurs étangs sélectionnés au préalable. Ces étangs varient par leur environnement proche, en étant entourés de **cultures agricoles**, de **milieux urbanisés** ou de **milieux forestiers**. Pour l'instant, les données sont majoritairement collectées et **en cours d'analyse**. Ainsi, je n'ai pas pu les inclure dans ce manuscrit. Nous avons néanmoins donné une première structure à l'analyse de nos données, qui correspondent à des prélèvements de larves de moustique durant l'été 2022. Ces données seront complétées et exploitées en **collaboration** avec les différents partenaires du projet.

Les **conditions environnementales** sont un facteur crucial dans la formation des **capacités cognitives** des animaux. En particulier, la présence de **pollution chimique** d'origine anthropique peut affecter les processus neurologiques et les **états physiologiques** des individus. Ces altérations peuvent être mesurées par l'observation du comportement individuel en laboratoire, et l'évaluation des capacités cognitives peut donner un aperçu de la qualité de l'environnement lui-même. Dans ce chapitre, nous avons étudié l'association potentielle des conditions environnementales avec l'activité et les capacités cognitives des larves de moustique. Nos résultats ont révélé des différences remarquables en matière **d'apprentissage** et **d'activité** entre les deux genres identifiés, les larves **d'*Anopheles*** et de ***Culex*** démontrant toutes deux la capacité de s'habituer à un stimulus aversif. Les **conditions d'élevage** ont eu un impact significatif sur ces performances, les individus élevés dans la **nature** présentant de meilleures capacités d'apprentissage et une activité accrue par rapport aux individus élevés en **laboratoire**. En outre, nous avons observé des différences contrastées entre les sites, qui sont discutées plus en détail. Comme ce travail fait partie d'un projet régional, la comparaison avec des données supplémentaires sur les caractéristiques chimiques de chaque étang collecté nous permettra de corréler nos différences

comportementales avec la **présence de polluants**. L'évaluation des capacités cognitives des larves de moustique pourrait être un outil avantageux pour estimer la qualité des écosystèmes d'eau douce.

Dessart M, Lazzari C, Guerrieri F (2024) Environmental conditions affect activity and cognition of mosquito larvae, but not responsiveness to visual stimuli. Manuscript in preparation.

Environmental conditions affect activity and cognition of mosquito larvae, but not responsiveness to visual stimuli

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Keywords: habituation, learning, locomotion, eco-ethology, *Culex, Anopheles*

Abstract

Environmental conditions are a crucial factor in shaping the cognitive abilities of animals. In particular, the presence of anthropogenic chemical pollution may affect neural processes and the physiological states of individuals. These alterations can be measured through the observation of individual behaviour in the laboratory, and evaluating cognitive abilities can provide insight into the quality of the environment itself. This study aimed to investigate the potential association between environmental conditions and the activity and cognitive abilities of mosquito larvae. The results revealed remarkable differences in learning and activity between the two genera identified, with both *Anopheles* and *Culex* larvae demonstrating the ability to habituate to an aversive stimulus. The rearing conditions had a significant impact on these abilities, with wild-reared individuals exhibiting better learning abilities and increased activity compared to laboratory-reared individuals. Additionally, we observed contrasting differences between sites, which are discussed in detail. As this work is part of a regional project, the comparison with additional data from the chemical characteristics of each of the ponds collected will enable us to correlate our behavioural differences with the presence of pollutants. Assessing the cognitive abilities of mosquito larvae could be a valuable tool for estimating the quality of freshwater ecosystems.

Introduction

Environmental conditions influence the development and cognitive abilities of animals (Papaj and Lewis 1993; Pasquini et al. 2024; Monchanin et al. 2024). For example, the abundance of predators in a given habitat may affect the ability to detect and actively avoid them, including learning from individual experiences and modifying future behaviour in an adaptive manner (Evans et al. 2019). The anthropogenic perturbation of natural habitats by factors, such as chemical pollution by agrochemicals, may negatively affect neural processes associated with information acquisition and processing, having as a potential consequence a degradation of cognitive performances. These sub-lethal effects on organisms and their impact on trophic networks are not always observable or easy to evaluate, but they could be good indications of the quality of ecosystems.

Eco-ethology is the science of the effects of animal behaviour on animal environment and the effects of animal environment on animal behaviour, with the purpose to find out how natural selection within this control loop has formed and moulded animals (Krebs and Davies 1978). In a conservation perspective, this approach can be applied to assess the status of ecosystems and guiding eventually conservation policies (Buchholz 2007).

The aim of our study was to evaluate the potential value of mosquito larvae as biological models for getting insights about the quality of aquatic ecosystems, using an eco-ethological approach. Mosquitoes are aquatic insects during their juvenile life. Female lay their eggs in water bodies which can much differ in terms of size, volume, chemical attributes, vegetation, presence of predators, etc. Throughout their postembryonic growth, mosquito larvae must adjust their behaviour to the environmental conditions in which they develop, including to learn to react or not to stimuli representing either predators or harmless elements (e.g. Baglan et al 2017).

Mosquito larvae spend most of their time suspended at the water surface, but when a moving object casts its shadow on the water, they display a stereotyped startle response, which consist of detaching from the surface, vigorous wiggling and plunging. The biological meaning of this reaction is that the shadow would be perceived as a

potential danger, i.e. a predator. If the same shadow is repeatedly projected without having any negative consequence for the larva, the insect reduces its escape response or eventually stops responding. This diminution in the escape tendency is due to habituation, a form of non-associative learning, in other words, the larvae have learned that the shadow does not represent a danger (Baglan et al. 2017, Dessart et al. 2023). This learning process can be reproduced in the laboratory and used to accurately assess the cognitive abilities in mosquito larvae (e.g., Dessart et al. 2023). Recent work has shown that these abilities can be impaired by the presence in the water of certain chemicals (Baglan et al. 2018; Dessart et al. 2024a), validating the idea that evaluating cognition may shed light on the quality of the aquatic environment where these aquatic organisms develop.

In this work we investigated whether there exists a relationship between the habitat in which different species of mosquito larvae develop and their behaviour, in particular, their activity and cognitive abilities. For this, we collected insects in water bodies differing in their characteristics, i.e., landscape (forest, grassland or urban) and anthropic influence (pristine, urban or agricultural), and evaluated their behaviour in the laboratory. Some groups of larvae completed their development in the wild, whereas others were collected early in the life and kept in the laboratory to reach the last instar. The main goal of our study was shed light on how (1) rearing conditions and (2) habitat characteristics might affect learning abilities, locomotor activity and responsiveness to visual stimuli in mosquito larvae belonging to different species.

Material and methods

The approach of this study was the same of previous studies (Dessart et. al 2023, 2024a-b). Briefly, the habituation to the passage of a shadow was analysed to evaluate learning performance, activity and responsiveness in mosquito larvae collected in natural habitats. Individual responses were recorded in an automatic way, videorecorded and analysed using tracking software.

1. Insects and their habitats

Mosquito larvae were collected from five artificial basins located in the department of Indre et Loire, Region Centre-Val de Loire, France (Figure 1A). We defined two categories of habitat, according to the landscape and the exposition to pollutants. The first category included three basins, mostly surrounded by woody vegetation, composed of varied vegetation (e.g., reed beds, wet meadows, lily pads), hosting several species of Odonata and amphibians, with water of apparently good quality for life to develop. Those three ponds were *Étang du Val Joyeux* (47°32'24 N, 0°19'33 E), *Étang de Givry* (47°27'45 N, 0°25'31 E) and *Grand Étang* (47°19'45 N, 0°15'41 E). For the second category, we selected a 10-ha basin (*Étang de l' Archevêque*) (47°31'29 N, 0°51'18 E) surrounded by agricultural fields and with reduced grass vegetation. We also sampled a pond of approximately 10 m^2 situated in an urban garden (*Jardin François Sicard*) in the city of Tours (47°23'40 N, 0°41'34 E). These five sites were located in a 30 km-radius circle. A recent study conducted by colleagues on the same ponds in order to characterise the communities of Odonata present in each of them, revealed notable differences across sites (Baeta and Pincebourde, pers. comm.). The authors found only 8 to 10 species in the agricultural site, while in the three forest sites, 20 to 23 species could be sampled. This important reduction in dragonfly diversity in habitats submitted to strong anthropic influence, makes the selected sites appropriate for the search of other deleterious effects of the environment on aquatic organisms.

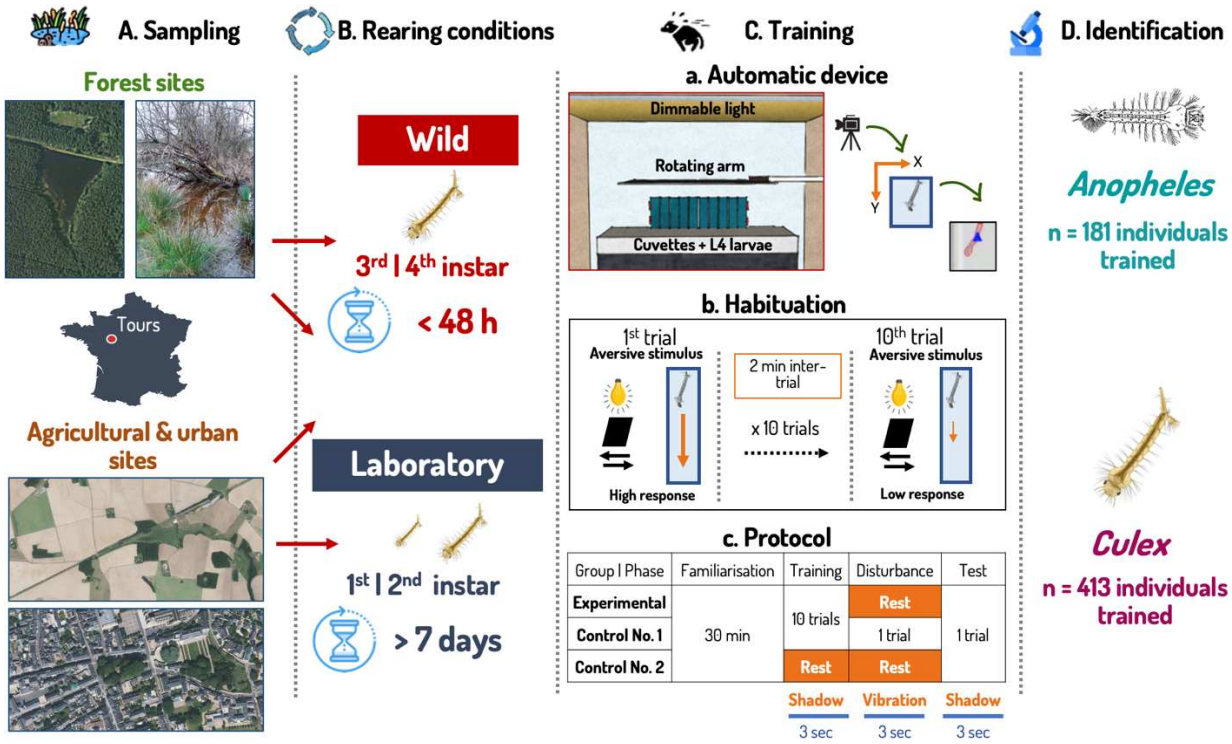

Figure 1: Schematic of the experimental processes. A) Mosquito larvae were collected from ponds located in the region Centre-Val de Loire, France. We classified these sites into Forest sites and Agricultural and urban sites. B) We collected individuals at the 3rd – 4th instar and maintained them less than 24 hours in the laboratory before to train them (wild-reared group) or we collected 1st or 2nd instar larvae and maintained them at least seven days in control conditions before training. C) Training procedure. a) We used an automatic device that consisted of a robotic arm creating a light contrast above cuvettes with individual larvae. We videorecorded all experiments and extracted subsequent data. b) Training consisted of the repetition of 10 trials, spaced by two minutes of inter-trial interval. c) We created three groups: experimental and two controls. d) We successfully identified two genera: *Anopheles* and *Culex* individuals.

To collect mosquito larvae, 10 trips were organized from the 11th of July 2022 to the 16th of August 2022. At each site, we scooped the water surface over a 1 m^2 sampling area using a 1 l recipient and gently transferred mosquito larvae into a 25 l recipient filled with natural water from the site until at least 100 individuals per basin were collected. Once in the laboratory, the larvae were transferred to 750 mL plastic containers and kept in a climate-controlled room at 25°C ± 2°C and under 12h:12h light:dark cycle (lights on at 8:00 am). Fourth-instar larvae were trained in the following two days after collection (group 1 "wild"– trained less than 48 hours after collection, Figure 1B), or we waited at least 7 days to train the remaining individuals (group 2 - trained over 7 days after collection, Figure 1B). For this second group, we collected 1st and 2nd instar larvae and reared them in the laboratory. Only fourth-instar larvae were used in all the experiments. All animals have been collected, reared and manipulated in accordance with the European Union ethical guidelines.

5. Identification

In the laboratory, an initial visual identification was made and individuals were placed in containers according to their genus (i.e., *Culex* or *Anopheles*, Figure 1D). This first assessment was straightforward as *Culex* larvae have a prominent siphon and angle their bodies downwards with the water surface, whereas *Anopheles* larvae have a short siphon and lie parallel to the water surface (Figure 1D). After training, individuals were kept until emergence and we performed a second morphological identification under a stereomicroscope, using the MosKeyTool database (Gunay et al. 2018). For *Culex*, we found at least two species: *Culex pipiens* and *Culex territans*, which differed in their siphon index (total length/diameter at the base) and the arrangement of their siphon setae. For *Anopheles*, we could not find any visible differences between the *Anopheles*, but it is most likely that the individuals belonged to the *Anopheles maculipennis* complex. The Moskeytool database did not go further than this complex. We also identified and excluded 4 individuals belonging to the genus *Culiseta*.

6. Apparatus

The experimental setup was similar to Dessart et al. (2023). It consisted of two light sources, a camera, and a servo mechanism that controlled the mechanical vibration and the projection of the shadow of a square piece of cardboard over twelve 1.5 ml spectrophotometer plastic tubes in which the larvae had been individually placed (Figure 1C). One light source, consisting of two LED panels (30 cm x 30 cm), was located at 0.3 ± 0.1 cm above the cuvettes, and provided a light intensity of 1500 $\mu W.cm^{-2}$ ± 100 $\mu W.cm^{-2}$ (Figure 1C). The second light source was an infrared backlight (880 nm) placed behind the cuvettes. In front of the cuvettes, a camera (acA 1300 – 60gc, Basler, Germany) equipped with an infrared high-pass filter (RG 850 Filter - 40.5 mm, Heliopan, US) recorded the experiments (for details, see Dessart et al., 2023). The projected shadow represented the motion of a potential flying predator and induced naive larvae to dive vertically, escaping from potential danger. After repeated presentations of the shadow, the escape response decreased due to habituation, a form of non-associative learning (Dessart et al. 2023). Two vibrators were placed on either side of the set of cuvettes and represented the motion of a potential fish predating the larvae (Figure 1C). This stimulus was used to assess dishabituation (see experimental procedure below).

7. Experimental procedure

The experimental procedure consisted of three groups trained in four consecutive steps: familiarisation, training, disturbance and test (Figure 1C). The experiments took place in the afternoon, from 12h to 19h. In an experimental room maintained at 25°C ± 0.5°C, larvae were carefully removed from their rearing container and individually placed in the cuvettes filled with dechlorinated tap water. The larvae were left undisturbed for 30 min (familiarisation, Figure 1C). During the training phase, the experimental group and the control N°1 group were exposed to the repeated presentation of the visual stimulus (shadow) for 10 consecutive times (trials), with a 2 min inter-trial interval (training, Figure 1C). The control N°2 group was left undisturbed during the training period. Two minutes after the 10th stimulus, the control N°1 was exposed to the mechanical stimulus, while the experimental group and control N°2 were left undisturbed (Disturbance, Figure 1C). After this phase, the three groups received another visual stimulation (Test, Figure 1C). After the test phase, we applied a final mechanical vibration for 3 seconds and removed from the database individuals that did not respond to the mechanical stimulus (27 individuals, Supplementary Table T1). We also removed individuals that were not visible enough for the tracking software to extract individual coordinates (23 individuals, Supplementary Table T1) or when individuals transformed into pupae during the experiment (3 individuals, Supplementary Table T1). In total, 597 individuals from 66 experiments were retained for the analysis.

8. Data analysis

Experiments were videotaped, and the trajectory of each individual was extracted using the software AnimalTA (Chiara & Kim 2023). This highly flexible video tracking program was particularly useful for individuals that spent most of their time at the water surface and could remain undetected with previous software. For all the trajectories, we applied a detection rate by comparing the number of frames successfully identified by the tracking algorithm to the theoretical maximum number of frames. All videos were analysed with a minimum detection rate of 80% (Supplementary Table T1). We then analysed 3 metrics by looking at individual trajectories. First, we assessed learning ability by extracting the behavioural response of individuals during the 3-seconds trial period of the shadow passage. With these data, we excluded individuals at the bottom of the

cuvette in the first frame of a trial (below 9/10 of the cuvette length, 6.6% of trials removed, Table 1). We then calculated the variable vertical distance (VD) as the vertical downward distance travelled by each individual during the 3 seconds stimulus onset (Dessart et al. 2023). Using VD, we excluded individuals that travelled up to the top of the cuvette during a trial (i.e., that travelled more than 10 mm upwards, 2.7% of trials removed, Supplementary Table T2).

The second set of metrics corresponded to the spontaneous locomotor activity of the individuals during the inter-trial intervals. As these data did not include a stimulation period, we did not apply a filter. We calculated the absolute distance (AD), as the sum of the individual displacements, regardless of the direction of movement (upward or downward), and decomposed this variable into 4 components: the average velocity (AV) (mm/s) corresponded to the average distance travelled in 1 second; the maximum velocity (MV) (mm/s) corresponded to the maximum distance travelled in 1 second per individual; the time spent moving (TM) corresponded to the proportion of time when the AD was greater than 1 mm; the number of diving events (DE) corresponded to the number of times an individual crossed both 1/3 and 2/3 of the cuvette length on its way in and out.

Finally, we developed another metric to quantify the distance travelled by individuals that responded on 1st trial, i.e., to encompass individual visual responsiveness (VR) on the 1st trial. By retaining only individuals that responded to the stimulus and comparing between treatments, we were able to gain insight into the ability to perform an escape response for the groups where the average AD was low. We created a function that measured, for each individual, the minimum number of frames to dive, from over a threshold of 1/8th (approximately 5 mm) of the cuvette length to below a threshold of 7/8th (approximately 35 mm) of the cuvette length. This metrics therefore only kept individuals that were near the top at the start of the 1st trial and had moved down near the bottom before the end of the trial. For *Anopheles*, we kept the response of 5 individuals from *Givry*, 4 individuals from *Archevêque* reared in the laboratory and 13 individuals from *Archevêque* reared in the wild. For *Culex*, we kept the response of 9 individuals from *Grand Étang* reared in the laboratory and 11 reared in the wild. We also kept the response of 19 individuals from *Tours* reared in the laboratory and 17 from the wild.

Statistical analyses

1. Data classification and filtering

We analysed the proportion of trials deleted by the filtering steps to avoid differences in vertical position between trials. We used a Chi-square goodness of fit test to verify that the average vertical position of individuals did not increase or decrease across trials.

9. Learning performance

We modelled the learning performance of individuals using the *Generalised Additive Model* to provide a visual estimate of the behavioural response across trials during the training period (Figure 2). Similar to our previous work, we chose the P-spline smoothing function as the best to describe our data (Dessart et al. 2023). We also compared the distance travelled during the 1st stimulus and the 10th stimulus and concluded that the individuals had not learned if the difference was not significant (Figure 3). For most sites, the unbalanced data set associated with opportunistic collection in the field was mainly responsible for the non-normality of the VD. Therefore, we used the Wilcoxon test for all the comparisons between the 1st and the 10th trials.

10. Habituation assessment

For all locations, we compared the response in the 10th trial, in the *disturbance* phase and in the *test* phase (Control N°1, Figure 4). As VD did not follow a normal distribution, we compared the response by using the Kruskal-Wallis rank sum test in conjunction with Bonferroni post hoc analysis. We used the same procedure to compare the 3 groups (i.e., control N°1, experimental and control N°2) at the test phase.

11. Spontaneous activity

We analysed the spontaneous activity by comparing individual activity during the inter-trial intervals. For each of the four metrics used, (i.e., average velocity (AV) (mm/s), maximum velocity (MV) (mm/s), time spent moving (TM, %), number of diving events (DE)), we applied a one-way ANOVA with one of the metrics as the response variable and

the site as a factor. We verified the variance homogeneity and the distribution of the residuals using the *simulateResiduals* function from the *DHARma* package (Hartig 2022). Pairwise comparisons were evaluated using the emmeans package with Tukey correction (Lenth 2021).

12. Visual responsiveness

Finally, we compared the responsiveness for each site using a one-way ANOVA with VR as the response variable and site habitat as a fixed factor. Similar to the analysis of the spontaneous activity, we tested for normality.

Results

1. Data classification and filtering

To verify that the deleted trials were not specific to a trial number (i.e., the average vertical position of the larvae remain similar across trials), we compared the proportion of deleted trials by the two criteria between trials (similar to Dessart et al. 2023). For each group, the deleted trials were not specific to a trial number (Supplementary Table T3).

13. Learning performance

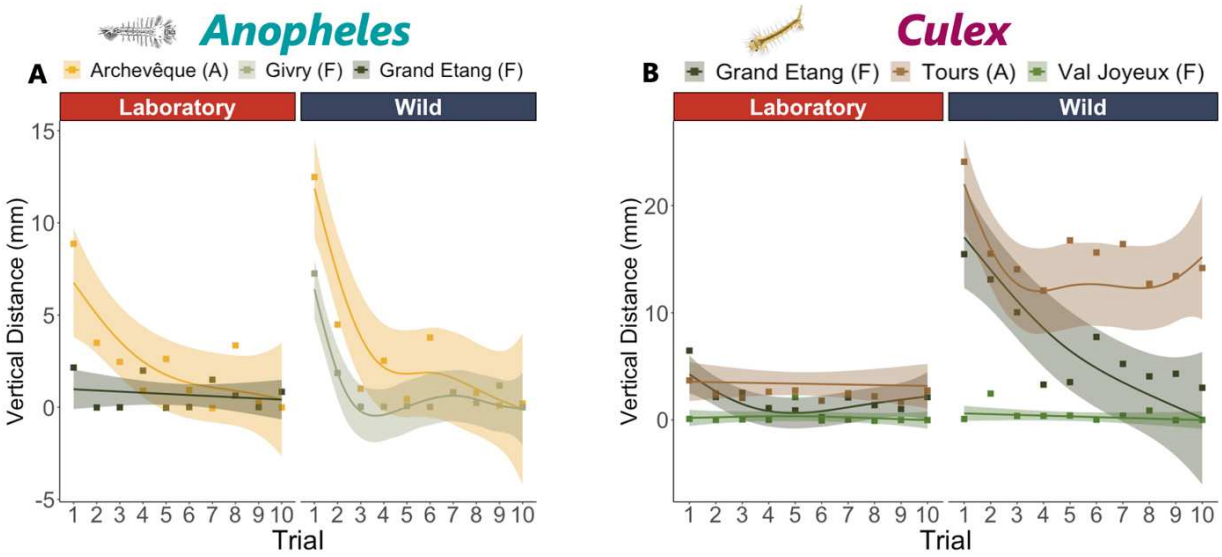

Figure 2: Behavioural response over the course of the training phase. Habituation curves for (A) *Anopheles* and (B) *Culex* larvae collected in *Archevêque* (yellow), *Givry* (pale green), *Grand Étang* (dark green), *Val Joyeux* (light green) and *Tours* (brown) and reared in the laboratory (red, left panel) or in the wild (blue, right panel). Vertical distance (in millimetre) corresponds to the distance travelled by one individual

during the stimulus period (3 sec), from the 1st to the 10th trial. Smoothing lines indicate the best fitted GAM model. Points indicate mean values. A = Agricultural site. F = Forest site.

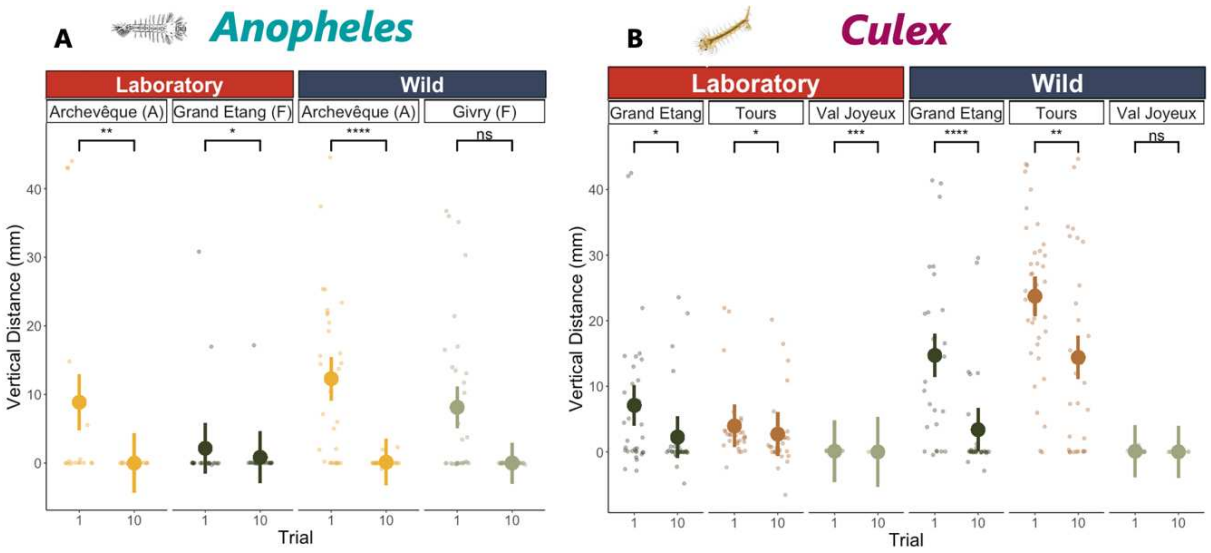

Figure 3: Learning performance. Comparison between the 1st and the 10th trial for (A) *Anopheles* and (B) *Culex* larvae. For each site, vertical distance in millimetre travelled by individuals responding to an aversive stimulus during the 1st and the 10th trial. Points indicate mean values and bars indicate +- 95% confidence intervals. A = Agricultural site. F = Forest site. NS, not significant; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Regarding the learning performance, two distinct patterns emerged for both *Anopheles* and *Culex* larvae (Figure 2). Individuals reared in the laboratory presented a limited behavioural response to the aversive stimulus compared to individuals reared in the wild. When behavioural responses were examined for each site and rearing condition, consistent differences were found (Figure 3). For *Anopheles*, VD was higher in the 1st vs the 10th trial for larvae collected in *Archevêque* (reared in the laboratory: Z = 7.43, P < 0.01; reared in the wild: Z = 23.97, P < 0.0001). For *Grand Étang,* the significant difference found must be taken with caution as the vertical distance travelled was very small for all stimuli (Z = 5.79, P = 0.02; Figure 3A). For larvae collected in *Givry*, although the differences between the 1st and 10th trial were consistent (1st trial: mean = 8.09, sd = 11.79; 10th trial: mean = 0.003; sd = 0.05), the non-parametric test applied showed no significant difference (Z = 2.87, P = 0.09) (Figure 3A). For *Culex*, VD was significantly higher in the 1st compared to the 10th trial for larvae collected in *Grand Étang* (in the laboratory: Z = 5.94, P = 0.01; in the wild: (Z = 18.71, P < 0.0001) and in *Tours* when reared in the wild (Z = 7.71, P < 0.01; Figure 3B). Although significant, the results found for larvae reared in the laboratory and collected in *Tours* and in *Val Joyeux* were mainly due to the

residual distances measured and did not provide a valuable difference (*Tours*: Z = 4.88, P = 0.03, *Val Joyeux* Z = 11.52, P < 0,001; Figure 3B). Finally, larvae collected in *Val Joyeux* and reared in the wild showed no differences between trials (Z = 1.83, P = 0.18; Figure 3B).

14. Habituation assessment

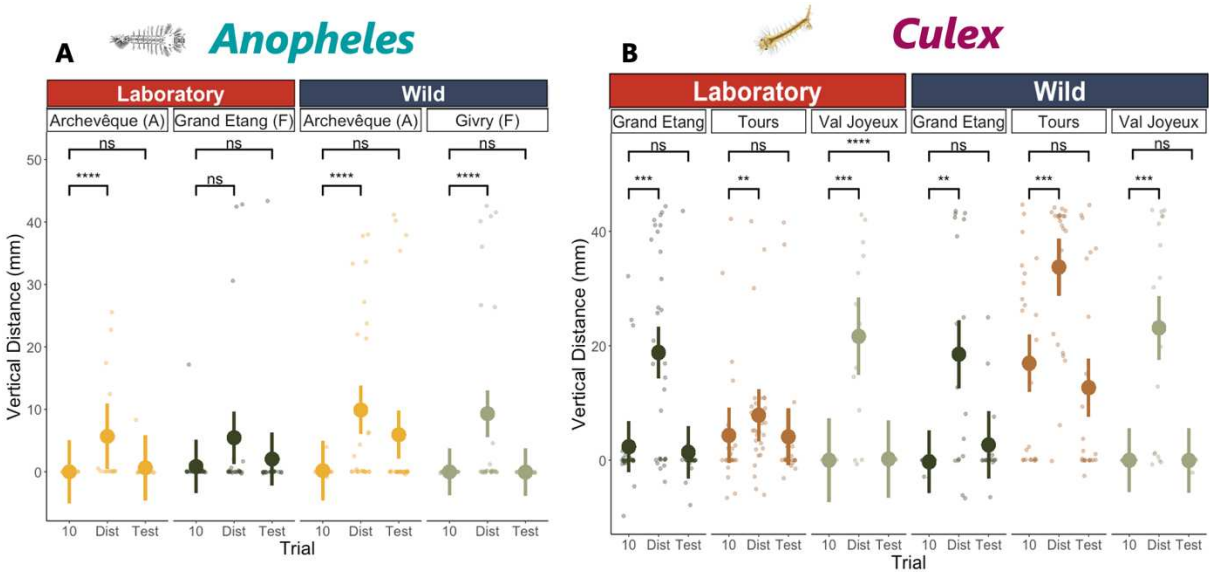

Figure 4: Dishabituation. Comparison between the 10th trial, the *disturbance* phase (Dist) and the *test* phase (Test) for (A) *Anopheles* and (B) *Culex* larvae. For each site, vertical distance in millimetre travelled by individuals responding to an aversive stimulus. Points indicate mean values and bars indicate +- 95% confidence intervals. A = Agricultural site. F = Forest site. NS, not significant; **P<0.01, ***P<0.001, ****P<0.0001.

As we did not find many larvae in each field session, we prioritised the training of the Control N°1 groups, followed by the experimental group and the Control N°2 (Figure 1). To look for evidence of dishabituation to occur, we compared the responses during the 10th trial, the disturbance and the test phase for the Control N°2 of each group (Figure 4). Overall, individuals responded strongly to the mechanical disturbance. The comparisons between the 10th trial and the disturbance were significant for all except the larvae reared in the laboratory and collected in *Grand Étang* (P = 1) (Figure 4). The other comparisons were significant (*Anopheles*: *Archevêque*: in the laboratory: P < 0.0001; in the wild: P < 0.001; *Givry*: P < 0.001; *Culex*: *Grand Étang*: P < 0.001; P < 0.001; *Tours*: P = 0.03; P < 0.01; *Val Joyeux*: P < 0.01; P < 0.001; Figure 4). However, the response in the test phase did not increase when compared to the test trial. Although one comparison

appeared significant (*Val Joyeux*: in the laboratory: P < 0.001), this was due to the residual values of small displacements for individuals and the small number of individuals kept along with non-parametric analysis. All other results did not show significant comparison (*Anopheles*: *Archevêque*: in the laboratory: P = 1; in the wild: P = 0.99; *Grand Étang*: P = 0.12; *Givry*: P = 1; *Culex*: *Grand Étang*: P = 1; P = 0.12; *Tours*: P = 1; P = 0.94; *Val Joyeux*: P = 0.13; Figure 4).

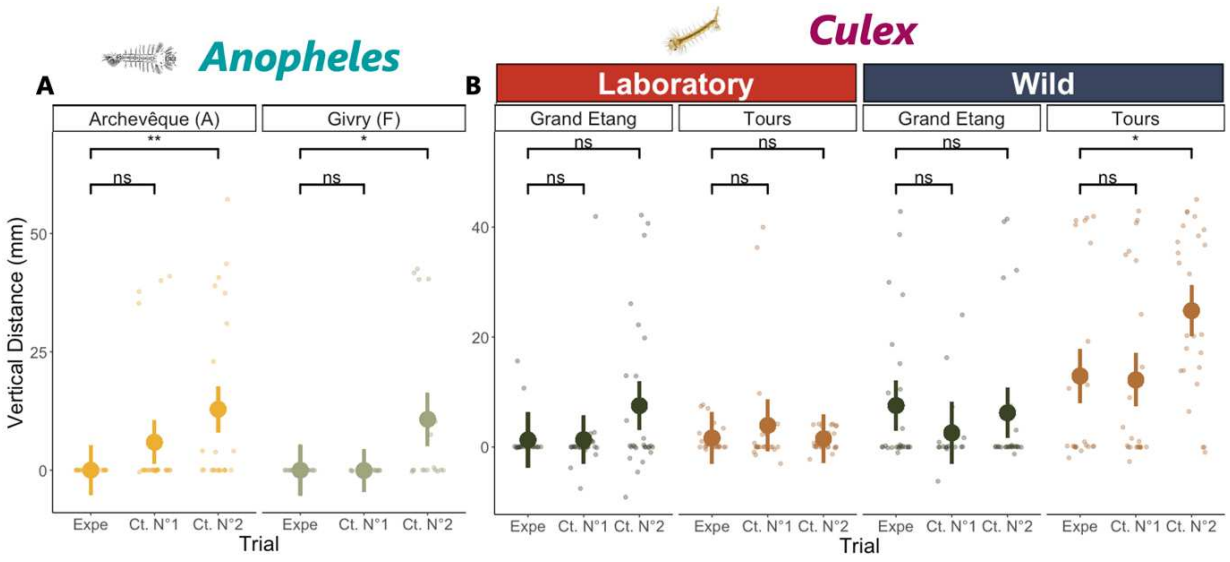

Figure 5: Test phase. Comparison between the test phase for the three experimental groups for (A) *Anopheles* and (B) *Culex* larvae. Experimental group: Expe; Control N°1: Ct. N°1; Control N°2: Ct. N°2. For each site, vertical distance in millimetre travelled by individuals responding to an aversive stimulus during the 1st and the 10th trial. Points indicate mean values and bars indicate +- 95% confidence intervals. A = Agricultural site. F = Forest site. NS, not significant; *P<0.05, **P<0.01.

Finally, by comparing the test phase between the three groups when data were available, we found a general increase in response between the experimental group and the Control N°2, evincing a possible contextual effect of the apparatus (*Anopheles*: *Archevêque*: P.= 0.022; *Givry*: P = 0.08; *Culex*: *Grand Étang*: P = 0.67; P = 1; *Tours*: P = 1; P = 0.09, Figure 5).

15. Spontaneous activity

To analyse spontaneous activity, we calculated four metrics indicating average and maximum velocity (AV and MV), the time spent in movement (TM) and the number of dives (DE) at the individual level. *Anopheles* larvae presented several significant differences as shown in Figure 6 (AV: ANOVA $F(3, 102) = 11.76$, $P < 0.0001$; MV: ANOVA $F(3, 176) = 8.38$, $P < 0.0001$; TM: ANOVA $F(3, 102) = 7.96$, $P < 0.0001$; DE: ANOVA $F(3, 102) = 9.45$, $P < 0.0001$). Specifically, larvae collected at *Archevêque* were the most active, regardless of their rearing condition, while larvae reared in a forest site were less active (wild-reared larvae from *Archevêque – Grand Étang*: AV: $P < 0.0001$; MV: $P < 0.0001$; TM: $P = 0.83$; DE: $P < 0.01$; wild-reared larvae from *Archevêque – Givry* : AV: $P < 0.0001$; MV: $P < 0. 0001$; TM: $P = 0.10$; DE: $P < 0.001$; Figure 6).

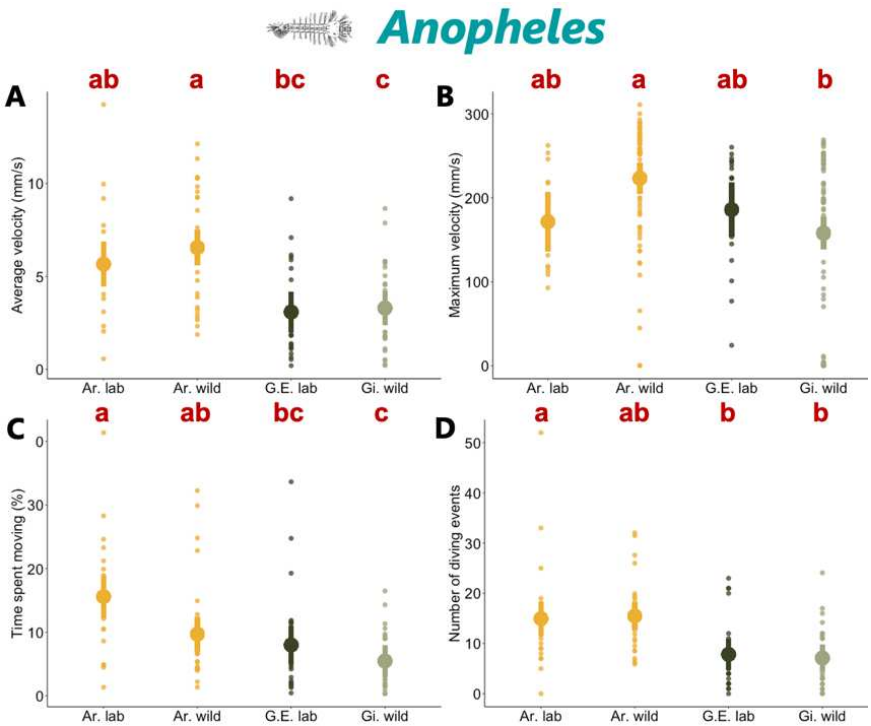

Figure 6: Spontaneous locomotor activity for *Anopheles* larvae. For each panel, larvae were collected in *Archevêque* (Ar., yellow), *Grand Étang* (G.E.,, dark green) and *Givry* (Gi., pale green). Lab = laboratory-reared larvae and wild = wild reared larvae. A) Average velocity (mm/s). B) Maximum velocity (mm/s). C) Average time spend moving (%). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Different letters above bars indicate significant differences between sites (ANOVA).

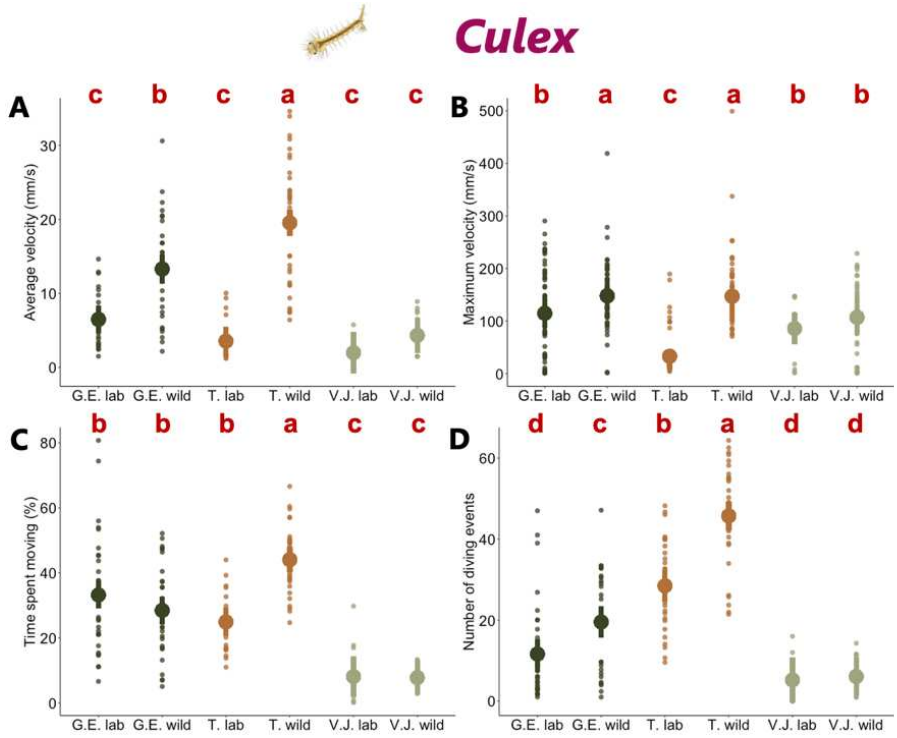

Figure 7: Spontaneous locomotor activity for *Culex* larvae. For each panel, larvae were collected in *Grand Étang* (G.E., dark green), *Tours* (T., brown) and *Val Joyeux* (V.J., light green). Lab = laboratory-reared larvae and wild = wild reared larvae. A) Average velocity (mm/s). B) Maximum velocity (mm/s). C) Average time spend moving (%). D) Number of diving events. Points indicate mean values and bars indicate +- 95% confidence intervals. Different letters above bars indicate significant differences between sites (ANOVA).

For *Culex* larvae, there were significant differences between sites for each of the four variables observed (AV: ANOVA $F(5, 157) = 47.61$, $P < 0.0001$; MV: ANOVA $F(5, 403) = 48.98$, $P < 0.0001$; TM: ANOVA $F(5, 157) = 34.93$, $P < 0.0001$; DE: ANOVA $F(5, 157) = 60.28$, $P < 0.0001$; Figure 7). Larvae reared in the wild were more active than larvae reared in the laboratory for *Tours* ($P < 0.0001$ for all), and for *Grand Étang* (AV: $P < 0.0001$; MV: $P < 0.01$; TM: $P = 0.58$; DE: $P = 0.03$; Figure 7), but not for *Val Joyeux* ($P > 0.07$ for all). In addition, wild-reared larvae collected in *Tours* were more active than those reared in *Grand Étang* and the least active were those reared in *Val Joyeux* (*Tours – Grand Étang:* AV: $P = 0.0001$; MV: $P = 1$; TM: $P < 0.0001$; DE: $P < 0.001$; *Grand Étang – Val Joyeux:* AV: $P < 0.0001$; MV: $P < 0.001$; TM: $P < 0.0001$; DE: $P < 0.001$; Figure 7).

16. Visual responsiveness

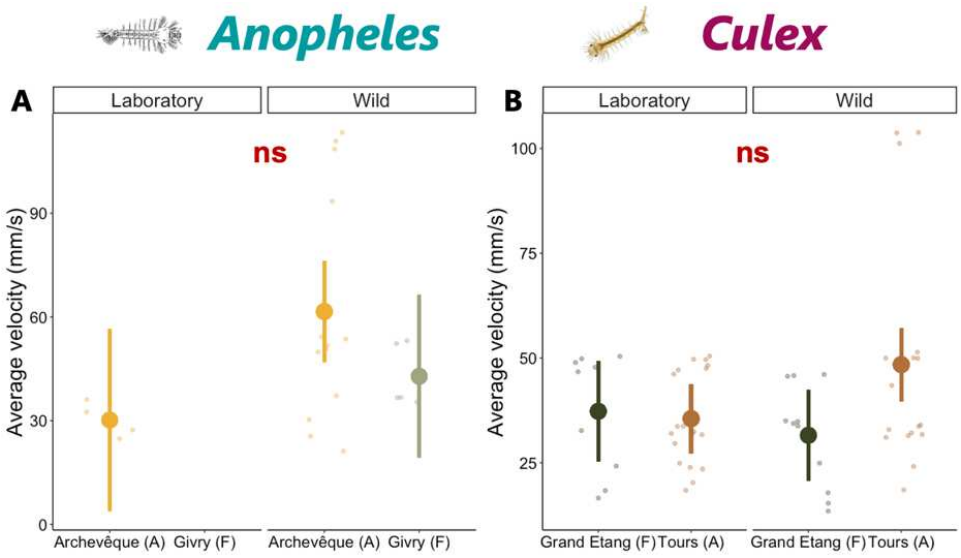

Figure 8: Visual responsiveness. Average velocity (mm/s) for (A) *Anopheles* and (B) *Culex* larvae collected in *Archevêque* (yellow), *Givry* (pale green), *Grand Étang* (dark green) and *Tours* (brown). Values corresponded to the average velocity to travel ¾ of the cuvette length during the first trial. Points indicate mean values and bars indicate +- 95% confidence intervals. NS, not significant.

Finally, we designed an algorithm that took only the first trial, i.e., the first encounter with the aversive stimulus, and kept only the individuals that were at the top of the cuvette at the beginning of the trial and moved to the bottom of the trial during the stimulus period (see Data analysis in the *Material and method* section). We then compared the average velocity of each site independently of the rearing condition. For each species, there were no significant differences between sites (*Anopheles*: ANOVA $F(2, 19) = 2.43$, $P = 0.11$; *Culex*: ANOVA $F(3, 52) = 2.35$, $P = 0.08$; Figure 8).

Discussion

Genus	Habitat	Site	Predation	Habitat	Pollution	Learning	Activity	Responsiveness
Culex	Urban	Tours	Low	Low	High	High	High	High
Anopheles	Agricultural	Archevêque	Low	Low	High	High	High	High
Culex	Forest	Val Joyeux	High	High	Int.	Low	Low	?
Anopheles	Forest	Givry	High	High	Low	High	Low	?
Culex	Forest	Grand Etang	High	High	Low	High	Int.	High
Anopheles	Forest	Grand Etang	High	High	Low	Low	Low	?

Table 1: Resume of the effects per site. General synthesis of the characteristics estimated for the different environments sampled, along with the main effects observed on mosquito behaviour. Predation indicated the main level of predation likely to be encountered by mosquito larvae. Habitat referred to the estimated relative abundance of the aquatic vegetation. Pollution indicated the estimated level of anthropic environment surrounding the pond. The Learning column gave the average learning performance. The Activity column referred to the main activity level. Responsiveness indicated the average visual responsiveness. Int. = intermediate. ? = unknown.

In this study, we adopted an eco-ethological approach to examine how the rearing conditions and habitat type may affect the behavioural performance of two genera of mosquito larvae, collected from ponds in the Centre Val de Loire region. The selected ponds varied in their landscape and potential presence of agrochemicals, potentially influencing the behaviour of mosquito larvae (Table 1). Contrary to what we expected, the level of anthropisation did not have a clear effect on the behavioural traits observed, but evinced an interaction with other characteristics of the habitat, as the abondance of aquatic vegetation (habitat structuration in the Table 1) and predator abondance, estimated from the presence of dragonflies according to data obtained by Baeta and Pincebourde (pers. comm.). Larvae proceeding from the two ponds most exposed to human influence, *Tours* (urban) and *Archevêque* (agricultural) evinced increased activity and habituation than other groups. Yet, the difference between these two types of habitats also involved the presence or absence of aquatic vegetation and predators, making difficult to discriminate individual effects (Table 1). Even though one can hypothesize that chemical pollution would have a deleterious impact on the nervous

system and eventually in larval behaviour, we do not know the behaviour of larvae of the same environment, but not exposed to pollutants. It is possible that activity is more determined by the presence or absence of vegetation providing refuge, than by chemical stressors. The same reasoning can be applied to learning performances.

Genus	Rearing condition	Habitat	Learning	Activity
Anopheles	Laboratory	Agricultural	High	High
Culex	Laboratory	Agricultural	Low	Int.
Anopheles	Laboratory	Forest	Low	Low
Culex	Laboratory	Forest	Int.	Low
Anopheles	Wild	Agricultural	High	High
Culex	Wild	Agricultural	High	High
Anopheles	Wild	Forest	High	Low
Culex	Wild	Forest	Int.	Int.

Table 2: Resume of the effects per rearing condition and habitat type. Synthesis of the main effects for the type of rearing condition and type of habitat. The Learning column gave the average learning performance. The Activity column referred to the main activity level. Responsiveness indicated the average visual responsiveness. Int. = intermediate.

Concerning the response of the different species found in the field; both *Anopheles* and *Culex* evinced similar abilities for non-associative learning. The activity of larvae belonging to both genera also remained similar, and varied similarly with the habitat. Our results indicated that the rearing conditions impacted individual abilities (Table 2). Notably, wild-reared individuals exhibited better learning abilities and higher activity. In contrast, larvae collected in the field but reared for a minimum of seven days in the laboratory showed reduced learning abilities and a relatively lower locomotion during training. In addition, larvae collected from either the agricultural or the urban site demonstrated robust learning abilities, alongside the most intense locomotor activity. Responses of larvae collected in ponds neighbouring forest sites were heterogeneous, thus we cannot describe a general behavioural pattern. Regardless of their rearing condition, both species may or may not show a decrease in their response to the stimulus over time.

To go further, we developed an algorithm that discerned whether individuals had responded to the first stimulus or not. For the responding individuals, we compared the average velocity and found no significant influence of rearing condition or site of origin on their velocity. This analysis confirmed that individuals from all sites and rearing conditions were able to perceive the stimulus, escaping the simulated aerial predator, and were able to travel from the top to the bottom part of the cuvette. In the groups with a small difference between the response at the 1st and the 10th trial and a low activity, the VR variable showed that a minority of individuals could perform an escape response at the same amplitude as other groups, but the majority did not. In consequence, the not-responding individuals should have a higher behavioural response threshold than the others, but may still be able to detect the stimulus.

In the ponds, larvae experienced the conditions of their natural habitats across several instars. They had to secure food, potentially escape predators and seek shelters in the vegetation (Clements 1999). By contrast, larvae collected at the first larval stage did not face the same challenges for long time, potentially limiting their experience with aversive stimuli. The marked contrast between the behavioural responses of individuals reared in the wild or in the laboratory may be accounted by the robust behavioural plasticity from mosquito, as has already been demonstrated in both larvae (Juliano and Stoffregen 1994; Ferrari et al. 2007; Diniz et al. 2017; Roberts 2018; Wooding et al. 2020; Pientrantuono et al. 2021) and adults (Lyimo and Ferguson 2009; Lefèvre et al. 2009; Takken and Verhulst 2013; Wolff and Riffell 2018; Carrasco et al. 2019).

Using controls groups, we finally investigated the cognitive abilities of trained individuals. Notably, we noticed a robust response for all conditions during the disturbance phase, which provides evidence to rule out motor fatigue. This finding is consistent with our previous results (Dessart et al. 2023). It should be mentioned that *Anopheles* larvae collected at *Archevêque* and reared in the wild demonstrated a stronger response during the test phase than in the 10th trial. In addition to the brief interval between the 10th trial and the test phase (i.e., 4 minutes), these results suggest a low probability of sensory adaptation. In this study, we aimed at eliminating biases from the field by training individuals in a controlled environment using an artificial device. Our intent was to encompass all the environmental variability by the different rearing conditions. However, differentiating between environmental and genetic factors was not

possible, and we need subsequent phase of research to disentangle these effects. One more convenient way would be to collect individuals from different habitats, examine their cognitive abilities and breed each population in the laboratory, to assess the cognitive abilities of the next generation.

In addition, we encountered a strong inter-individual variability in our study in comparison with previous ones on mosquito larvae (Dessart et al. 2023). While the availability of food was unrestricted in the laboratory, we were unable to control the amount and quality of nutrients present in natural habitat, climatic changes, the presence of contaminants as well as the real abundance of Odonata predators or other types of predators (Vinogradov et al. 2022).

In this study, we explored the cognitive abilities of mosquito larvae exposed to different environments, by using an eco-ethological approach. Further investigations are required to identify which physical and chemical parameter promote or hinder behavioural response, learning, visual responsiveness and locomotor activity. A separate analysis on the same ponds as part of the regional project COMPORTATE identified diverse Odonata communities present in the year 2021 (Baeta and Pincebourde, pers. comm.). As indicated in the Material and Methods section, this study revealed a notable decrease in the biodiversity of dragonflies that correlates with the human impact on aquatic habitats. On the other hand, our study reveals differences in activity and cognitive abilities of mosquito larvae that also suggest an influence of human activities; yet, these effects are not clear, being linked with other characteristics of the environment. Taking together, these two studies unravel a situation where both preys (mosquitoes) and predators (dragonflies) belonging to the same trophic network are impacted by environmental modifications. To what extent these effects compromise the health of ecosystems, still needs additional effort and data collection. Yet, simple approaches as evaluating the behaviour of mosquito larvae in the habituation paradigm seems to be a valuable tool for detecting anomalies in the quality of aquatic environments.

Acknowledgements

Financial support for this work was granted by Region Centre-Val de Loire, France APR IR 2020 COMPORTATE. COMPORTATE is supported by Réseau MiDi - Milieux et Diversité, Pole DREAM - French Water Team, Zone Atelier de la Loire – LTER France Loire, Réseau ENTOMOCENTRE, Département d'Indre-et-Loire - Direction de l'Attractivité des Territoires (France).

We thank Nathalie Gassama, Renaud Baeta, Arnaud Leroy, Laetitia Fougère, Emilie Destandeau Ali Fadel and Sylvain Pincebourde, for fruitful discussions on the ponds. Martin Dessart expresses his gratitude to Agathe Costé and Hugo Desriac for their help in the field work.

Author contributions

Martin Dessart: Conceptualization**,** Data curation, Formal analysis, Investigation, Methodology, Validation, Software, Visualization, Writing – original draft, Writing – review & editing. **Claudio R. Lazzari:** Conceptualization, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. **Fernando J. Guerrieri:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material

Replicate	Species	Location	Rearing	Group	ID number	Detection rate	Vertical length (px)	Comment
1	*Culex*	Tours	<48 h	Ct. N°1	6	0.93	405.8	ID#5, ID#6, ID#7, ID#8 identified as Culiseta
2	*Culex*	Tours	<48 h	Ct. N°1	5	0.93	402.3	ID#1 to ID#5 did not respond to vibration
3	*Culex*	Tours	<48 h	Ct. N°1	10	0.85	400.3	
4	*Culex*	Grand Etang	> 7 d	Ct. N°1	10	0.87	396	
5	*Culex*	Tours	<48 h	Expe	7	0.9	404.2	ID#4 and ID#9 did not respond to vibration, ID#10 dead
6	*Culex*	Grand Etang	<48 h	Ct. N°1	8	0.98	401.4	ID#1 and ID#5 did not respond to vibration
7	*Anopheles* *Culex*	Archevêque Grand Etang	> 7 d > 7 d	Expe	1 7	0.82	412.6	
8	*Culex*	Grand Etang	> 7 d	Ct. N°1	10	0.93	391.1	
9	*Culex*	Tours	> 7 d	Expe	10	0.9	410.6	
10	*Culex*	Tours	> 7 d	Ct. N°2	10	0.93	402.1	
11	*Culex*	Tours	> 7 d	Ct. N°1	10	0.93	403.8	
12	*Culex*	Tours	> 7 d	Expe	10	0.92	403.6	
13	*Culex*	Tours	> 7 d	Ct. N°1	10	0.97	404.7	
14	*Culex*	Tours	> 7 d	Ct. N°2	10	0.97	408.1	
15	*Culex*	Tours	> 7 d	Expe	9	0.89	404	ID#5 did not respond to vibration
16	*Anopheles* *Culex*	Grand Etang Grand Etang	> 7 d <48 h	Expe	3 7	0.99	396.2	
17	*Anopheles*	Grand Etang	> 7 d	Ct. N°1	9	0.94	410.9	ID#10 not visible
18	*Culex*	Grand Etang	> 7 d	Expe	8	0.99	401.2	ID#10 not visible, ID#2 did not respond to vibration
19	*Culex*	Grand Etang	> 7 d	Expe	10	0.97	401.8	
20	*Anopheles*	Archevêque	<48 h	Ct. N°1	10	0.99	409.2	
21	*Anopheles*	Archevêque	<48 h	Expe	8	/	412.9	ID#3 dead, ID #10 not visible
22	*Anopheles*	Archevêque	<48 h	Ct. N°1	8	/	411.3	ID#8 did not respond to vibration, ID#6 not visible
23	*Anopheles*	Archevêque	<48 h	Expe	7	/	411.3	ID#2, ID#3, not visible, ID#7 did not respond to vibration
24	*Anopheles*	Archevêque	<48 h	Ct. N°1	9	/	417.5	ID#5 dead
25	*Anopheles*	Archevêque	<48 h	Ct. N°2	8	/	401.3	ID#3 dead, ID#10 not visible
26	*Anopheles*	Archevêque	<48 h	Ct. N°2	10	0.87	407.1	
27	*Culex*	Grand Etang	> 7 d	Ct. N°1	10	0.99	402.2	
28	*Culex*	Grand Etang	> 7 d	Ct. N°2	10	0.85	402.3	
29	*Culex*	Grand Etang	> 7 d	Ct. N°2	10	0.99	406.3	
30	*Anopheles* *Culex*	Grand Etang Grand Etang	> 7 d <48 h	Ct. N°1	3 5	0.83	406.5	ID#1 not visible and ID#3 did not respond to vibration
31	*Culex*	Grand Etang	> 7 d	Ct. N°2	9	1	404.5	ID#10 not visible
32	*Anopheles*	Archevêque	> 7 d	Ct. N°1	10	0.94	408.3	
33	*Anopheles*	Archevêque Givry	> 7 d <48 h	Ct. N°1	5 5	0.88	413.4	

Replicate	Species	Location	Rearing	Group	ID number	Detection rate	Vertical length (px)	Comment
34	*Anopheles*	Givry	<48 h	Ct. N°1	9	0.99	407.2	ID#3 transformed in pupae
35	*Culex*	Val Joyeux	<48 h	Ct. N°1	10	0.98	399.5	
36	*Anopheles*	Givry	<48 h	Ct. N°1	9	0.99	400.2	ID#8 not visible
37	*Culex*	Val Joyeux	<48 h	Ct. N°1	10	0.99	393.4	
38	*Culex*	Val Joyeux	<48 h	Expe	10	0.98	398	
39	*Anopheles*	Givry	<48 h	Expe	10	0.99	414.1	
40	*Culex*	Val Joyeux	<48 h	Expe	10	0.99	391.9	
41	*Anopheles*	Givry	<48 h	Expe	10	0.95	411.3	
42	*Anopheles* *Culex*	Grand Etang Grand Etang	> 7 d > 7 d	Ct. N°1	1 4	0.98	412.3	ID#1, ID#2, ID#4, ID#5, ID#6 not visible
43	*Anopheles*	Givry	<48 h	Ct. N°2	9	0.89	392.4	ID#1 not visible
44	*Culex*	Val Joyeux	<48 h	Ct. N°2	9	0.95	405.3	ID#3 not visible
45	*Anopheles*	Givry	<48 h	Ct. N°2	8	0.9	393.6	ID#1, ID#8 not visible
46	*Culex*	Val Joyeux	<48 h	Ct. N°2	10	0.99	404.4	
47	*Culex*	Grand Etang	<48 h	Expe	9	0.99	401.6	ID#3 transformed in pupae
48	*Culex*	Grand Etang	<48 h	Ct. N°2	9	0.93	402.1	ID#7 dead
49	*Culex*	Grand Etang	<48 h	Ct. N°1	10	0.99	403.2	
50	*Culex*	Grand Etang	<48 h	Expe	10	0.99	399.6	
51	*Culex*	Grand Etang	<48 h	Ct. N°2	10	0.99	395.6	
52	*Culex*	Grand Etang	<48 h	Ct. N°2	10	0.98	397.9	
53	*Anopheles* *Culex*	Givry Val Joyeux	<48 h > 7 d	Ct. N°1	5 5	0.89	408.6	
54	*Culex*	Val Joyeux	> 7 d	Ct. N°1	9	0.91	400.7	ID#9 transformed in pupae
55	*Anopheles*	Grand Etang	> 7 d	Ct. N°1	7	0.86	412.2	ID#1 and ID#10 position empty, ID#3 not visible
56	*Anopheles*	Archevêque	<48 h	Expe	7	/	407	ID#2, ID#4, ID#6 did not respond to vibration
57	*Anopheles*	Archevêque	<48 h	Ct. N°2	6	/	399.4	ID#7, ID#8, ID#9 not visible, ID#10 dead
58	*Culex*	Tours	<48 h	Ct. N°1	8	0.95	402.4	ID#1 and ID#2 did not respond to vibration
59	*Culex*	Tours	<48 h	Expe	9	0.95	407.2	ID#8 did not respond to vibration
60	*Culex*	Tours	<48 h	Expe	10	0.93	404.6	
61	*Culex*	Tours	<48 h	Ct. N°2	9	0.96	402	ID#2 did not respond to vibration
62	*Culex*	Tours	<48 h	Ct. N°2	9	0.95	392.1	ID#10 dead
63	*Culex*	Tours	<48 h	Ct. N°2	8	0.87	401.3	ID#2 dead, ID#10 not visible
64	*Anopheles*	Archevêque	> 7 d	Ct. N°1	3	0.99	407.2	only 3 individuals on this replicate
65	*Culex*	Tours	> 7 d	Ct. N°2	10	0.97	399.4	
66	*Culex*	Tours	> 7 d	Ct. N°1	10	0.99	398.5	Comment

Supplementary Table T1: Details for each experiment performed. Species represents the species collected. Each replicate represents 10 individuals trained during one session. Location corresponded to the site of collection. If the cell is divided in two, it means that we tested 5 individuals for one site and 5 for another site. Rearing column corresponded to the time spent in the laboratory (<48 hours or > 7 days). Group column corresponded to one of the three groups (Experimental, Control N°1, Control N°2) tested. ID number correspond to the number of individuals for each replicate. Detection rate was calculated as the ratio between the maximum frame number and the actual frame number identified by the tracking software. Vertical length was calculated as the difference between the maximum and the minimum individual position measured on each video by the tracking software.

Species	*Anopheles*				*Culex*						All
Rearing conditions	Laboratory-reared		Wild-reared		Laboratory-reared			Wild-reared			
Sites	**Grand Etang (F)**	**Archevêque (A)**	**Givry (F)**	**Archevêque (A)**	**Tours (A)**	**Grand Etang (F)**	**Val Joyeux (F)**	**Tours (A)**	**Grand Etang (F)**	**Val Joyeux (F)**	**Total**
Individuals trained	23	19	65	73	89	88	14	89	78	59	597
Trials per individuals	266	215	586	513	735	706	163	683	617	495	4979
Trials filtered by position	259	199	579	498	646	660	158	606	554	484	4643
% Trials removed	2.6%	7.4%	1.2%	2.9%	12.1%	6.5%	3.1%	11.3%	10.2%	2.2%	**6.7%**
Trials filtered by going up	252	188	573	487	643	645	155	575	526	478	4522
% Trials removed	2.7%	5.5%	1.0%	2.2%	0.5%	2.3%	1.9%	5.1%	5.1%	1.2%	2.6%
Total % Trials removed	5.3%	12.6%	2.2%	5.1%	12.5%	8.6%	4.9%	15.8%	14.7%	3.4%	**9.2%**

Supplementary Table T2: Summary of the filtering steps. For each species, 19 to 89 individuals were trained. When the individual's position was close to the bottom, the response to the trial was removed, accounting for a total of 6.7% of trials removed. The trajectory of an individual moving upwards during a stimulation was rare, representing only 2.6% of trials.

Species	*Anopheles*				*Culex*					
Rearing conditions	Laboratory-reared		Wild-reared		Laboratory-reared			Wild-reared		
Surrounding	Forest	Agricultural	Forest	Agricultural	City	Forest	Forest	City	Forest	Forest
Sites	Grand Etang (F)	Archevêque (A)	Givry (F)	Archevêque (A)	Tours (A)	Grand Etang (F)	Val Joyeux (F)	Tours (A)	Grand Etang (F)	Val Joyeux (F)
χ 2	8.29	9	6.38	5.38	7.91	11.98	7	12.2	8.3	7.71
df	11	11	11	11	11	11	11	11	11	11
P =	0.69	0.62	0.85	0.91	0.72	0.36	0.8	0.35	0.69	0.74

Supplementary Table T3: Similar to Dessart et al. (2023, 2024a, 2024b), we tested whether the number of trials deleted by the criterion depended on the trial number, by applying a chi-squared test to the deleted trials as a function of the trial number for each treatment. For all sites, the deleted trials were not specific to any trial number.

Chapitre 6 : Discussion

So it goes on. In the behaviorist's Umwelt the body produces the mind, and in the psychologist's world the mind builds the body. [...] And yet all these diverse Umwelten are harbored and borne by the One that remains forever barred to all Umwelten. Behind all the worlds created by Him, there lies concealed, eternally beyond the reach of knowledge, the subject -- Nature.

*Jakob **von Uexküll (1934)** A Stroll Through the Worlds of Animals and Men*

Table des matières

Retour sur les résultats principaux de la thèse

Les moustiques sont abhorrés, et à raison. En fonction de la région du monde dans laquelle nous vivons, les moustiques peuvent nous déranger, nous agresser, nous rendre malade, ou tout à la fois. Pourtant, leurs caractéristiques, notamment pendant leur phase larvaire, pourraient nous permettre d'acquérir une multitude d'informations sur la qualité de leur environnement. Durant ma thèse, je me suis intéressé à la caractérisation de l'apprentissage, de la mémoire et de la locomotion des larves de moustique en lien avec leur contexte écologique. J'ai tenté de comprendre comment l'environnement, notamment la présence de polluants, pouvait moduler les capacités de trois genres : les genres *Aedes*, *Culex* et *Anopheles*.

Dans le **Chapitre 2** (Dessart et al. 2023), j'ai d'abord proposé un nouveau dispositif permettant de mesurer le comportement des larves de moustique. Grâce à un système automatisé et un suivi vidéo, j'ai pu valider l'apprentissage par habituation chez *Aedes, Culex* et *Anopheles*, avec des différences importantes de réponses comportementales et de locomotions. Dans le **Chapitre 3**, je me suis intéressé à la persistance de la mémoire chez l'espèce *Aedes aegypti*. De manière surprenante, les larves de cette espèce ne retiennent une information visuelle en contexte de prédation que jusqu'à 2 heures. Même en augmentant l'intervalle entre les essais durant la phase d'entrainement, les larves ne présentent aucune trace de cette information 24 heures après l'entrainement. Dans le **Chapitre 4**, j'ai utilisé la même espèce que j'ai soumis à trois polluants, de manière unitaire ou en combinaison. Les larves ont été exposées aux polluants de manière chronique ou aigüe, et j'ai mesuré l'apprentissage, la mémoire et la locomotion des larves via le dispositif précédemment développé. Les trois composés ont montré un impact significatif sur nos trois paramètres, et j'ai également mis en évidence un effet cocktail des trois polluants aux doses mesurées directement dans l'eau. Enfin, dans le **Chapitre 5**, j'ai mesuré l'apprentissage, la locomotion et la sensibilité visuelle des larves du genre *Culex* et *Anopheles* que j'ai collecté en région Centre Val de Loire. J'ai comparé des conditions d'élevage en laboratoire ou en milieu naturel, dans des étangs et dans des contextes de pollution anthropique. J'ai montré

d'importantes différences entre les étangs, ainsi que des meilleures performances chez les individus élevés en milieu naturel.

Ensemble, ces résultats apportent un nouvel outil qui permet d'explorer l'apprentissage par habituation chez la larve de moustique et qui combine approche automatisée et maîtrise des paramètres de tests. Ces quatre séries d'expériences permettent aussi de dévoiler des informations fondamentales sur la cognition des larves de moustique, mettant en perspective leur capacité à percevoir et à fuir un prédateur, à se mouvoir dans un espace restreint, et à garder une information en mémoire via une approche comparée. Ce travail s'inscrit également dans un effort de caractérisation de l'effet de polluants sur les invertébrés d'eau douce et permet d'envisager l'utilisation de la cognition des larves de moustique comme indicateur des écosystèmes d'eau douce. Ces points sont discutés dans ce **Chapitre 6**.

Méthode quantitative pour étudier la cognition chez les larves de moustique

La première moitié de cette thèse a été consacrée au développement d'un **dispositif automatisé** qui permet de mesurer le comportement de larves de moustique, et qui a été utilisé par la suite dans chacune des quatre séries d'expériences. Ce dispositif possède plusieurs **avantages**, dont celui de pouvoir régler finement chacun des paramètres. D'abord, numériquement, via l'interface électronique Arduino qui pilote les moteurs vibrateurs et le bras robotisé. Cette interface permet de régler le nombre d'essais, le temps d'apparition, les intervalles de temps et la vitesse de rotation du bras. Ensuite, physiquement, via le réglage de l'intensité lumineuse et des distances entre les différents éléments, afin de représenter un prédateur plus ou moins saillant pour les larves. Enfin, les données extraites sont également très modulables, via l'utilisation de paramètres de standardisation. J'ai utilisé par exemple une moyenne glissante, un filtre passe-bas et des seuils de classification, mais il est envisageable de prendre également en compte d'autres critères. Par exemple, plusieurs études normalisent les données de distance par le « body length », la taille moyenne des individus, ou l'activité moyenne de

locomotion (Risse et al. 2017; Reiskind et Janairo 2018; Amer et al. 2022). Les analyses pourraient également utiliser la position sur l'axe X (horizontal) et l'ajouter dans nos modèles en covariable avec la position Y afin d'avoir une meilleure appréciation de l'activité des individus. Ces données pourraient permettre d'être plus précis, même si des essais préliminaires nous ont permis de répondre à nos questions sans l'ajout de ces réglages.

Cet aspect de réglage de notre système peut toutefois représenter un **inconvénient** méthodologique. En effet, l'intensité du stimulus doit être suffisamment forte pour une réponse comportementale chez la majorité des individus, mais pas trop forte et nécessiter plusieurs dizaines d'essais avant de voir une modification comportementale. Les résultats montrent également une variabilité inter-individuelle remarquable qui nécessite de répliquer chaque traitement et d'avoir un nombre d'individus conséquent. Par ailleurs, le nombre de répliques est aussi lié à l'ajout du premier filtre. Celui-ci élimine les réponses aux essais des individus situés au fond de la cuve en début d'essai. Ainsi, lorsqu'un individu a une locomotion très active, comme *Aedes aegypti*, le nombre d'essais éliminé moyen est d'environ 22% (Figure 1).

	Aedes aegypti	*Culex*	*Anopheles*
Nombre d'individus entrainés	1035	354	181
Nombre d'essais par individus	10501	2974	1595
1) Essais où l'individu est trop proche du bas	2302	241	45
Pourcentage d'essais éliminés par 1)	21.9%	8.1%	2.8%
2) Essais où l'individu se déplace vers le haut	77	93	37
Pourcentage d'essais éliminés par 2)	0.9%	3.4%	2.4%
Pourcentage total d'essais éliminés	22.7%	11.2%	5.1%

Figure 19: Résumé du nombre d'individus entrainés et de l'effet des filtres sur les données. Le premier filtre (1) permet d'éliminer les essais où un individu est déjà au fond de la cuve lorsque le stimulus se présente. Bien que l'individu perçoive le contraste visuel, il ne peut physiquement pas répondre, c'est-à-dire fuir vers le bas. Le second filtre (2) permet de supprimer les essais où l'individu se dirige vers le haut.

De plus, lors de la phase d'identification des individus sur la vidéo, réalisée grâce au développement d'un algorithme d'identification (tracking), certains détails expérimentaux ont pu diminuer la performance du tracking. Des bulles d'air contre les parois, un individu qui reste particulièrement immobile, sont autant d'exemples qui

nécessitent une manipulation minutieuse du dispositif. Il est important de souligner ici que l'utilisation du logiciel AnimalTA pour le **Chapitre 5** a permis une plus grande flexibilité d'utilisation et un meilleur seuil de détection (Chiara et Kim 2023).

En représentant un stimulus dans les voies visuelles et mécano-sensorielles, nous avons les configurations pour recréer les expériences déjà menées par Holmes en 1911 jusqu'à Harrison en 2021 (Holmes 1911; Harrison et Budenberg 2021). Ce système pourrait également manipuler le **contexte visuel**, comme l'ont déjà proposé Pientrantuono et al. (2021), avec la variation de la forme et des motifs du stimulus visuel ou l'apparition d'un fond d'écran différent situé au-dessus, derrière ou au-dessous des larves (Pientrantuono et al. 2021).

En somme, notre dispositif est peu couteux, et facile à reproduire. Le remplacement de l'appréciation subjective humaine à l'aide d'une caméra et d'un logiciel d'extraction des positions des individus permet de s'affranchir du **biais** expérimentateur. Un biais lié au choix des paramètres est certes toujours présent, mais celui-ci reste contrôlable, quantifiable et constant au cours du temps. En plus de gagner en reproductibilité, cette méthodologie permet un gain de temps, qui peut être précieux lorsque l'on travaille avec des espèces à durée de vie très courte. Lors de notre première étude qui comparait l'habituation chez *Aedes, Culex et Anopheles*, nous avions comparé deux variables réponses : l'une quantitative (distance verticale parcourue pendant le stimulus) et l'autre qualitative (index de performance). Cette dernière permet d'avoir une estimation rapide de la réponse comportementale des individus en établissant un seuil et en classant les réponses en deux catégories : l'individu répond ou non. Cette variable est très utilisée dans d'autres apprentissages classiques tels que le réflexe d'extension du proboscis chez la punaise ou l'abeille, ou le réflexe d'extension du labium-maxilles chez les fourmis (Guerrieri et d'Ettorre 2010; Vinauger et al. 2013; Giurfa 2015). Les deux variables employées ont également montré une forte similitude dans la première étude du **Chapitre 2**, et également lors de l'étude terrain du **Chapitre 5** (Figure 2).

Le choix d'analyser la distance parcourue par les individus permet d'avoir une **approche quantitative**. Celle-ci, recommandée par de nombreux auteurs pour les

études en écotoxicologie, permet ici de savoir comment un individu va modifier sa réponse à un stimulus au cours du temps (Bownik et Wlodkowic 2021). En réalité, il est plus probable que les individus modifient leur réponse face à un stimulus aversif de manière progressive, et non de façon absolue. Ainsi, via cette mesure, nous avons pu mesurer la modification du comportement à l'échelle individuelle.

Enfin, notre approche nous permet d'obtenir des informations sur la réactivité des individus, leur locomotion spontanée, ainsi que les zones où ils passent le plus de temps. Dans le **Chapitre 4**, nous avons montré qu'il était important de prendre en compte d'autres critères que la réponse au stimulus afin de mieux saisir l'étendue de l'effet des polluants. A chaque instant, un individu est soumis à un trade-off entre consommer de l'énergie pour se déplacer ou rester sur place. Lorsqu'un prédateur se présente, ce choix peut être crucial pour sa survie, et cette dernière est également modifiée si l'individu se déplace constamment (Lucas et al. 2001). Dans cette thèse, nous nous sommes particulièrement intéressés à la locomotion spontanée et à la réponse de fuite, mais notre dispositif permet **d'évaluer d'autres types de comportements** via l'ajout de nouveaux stimuli, comme de la nourriture en surface ou en profondeur, une modification de la température de l'eau, et permet également d'estimer un budget-temps à l'échelle individuelle. Toutefois, même si en milieu naturel, un individu est soumis à une multitude de stimuli, il se retrouve ici dans un **dispositif artificialisé**. La cuve de spectrophotométrie est loin de représenter l'habitat naturel d'une larve de moustique et peut engendrer du stress. Ainsi, les larves ne peuvent pas forcément exprimer toute l'étendue de leur répertoire comportemental.

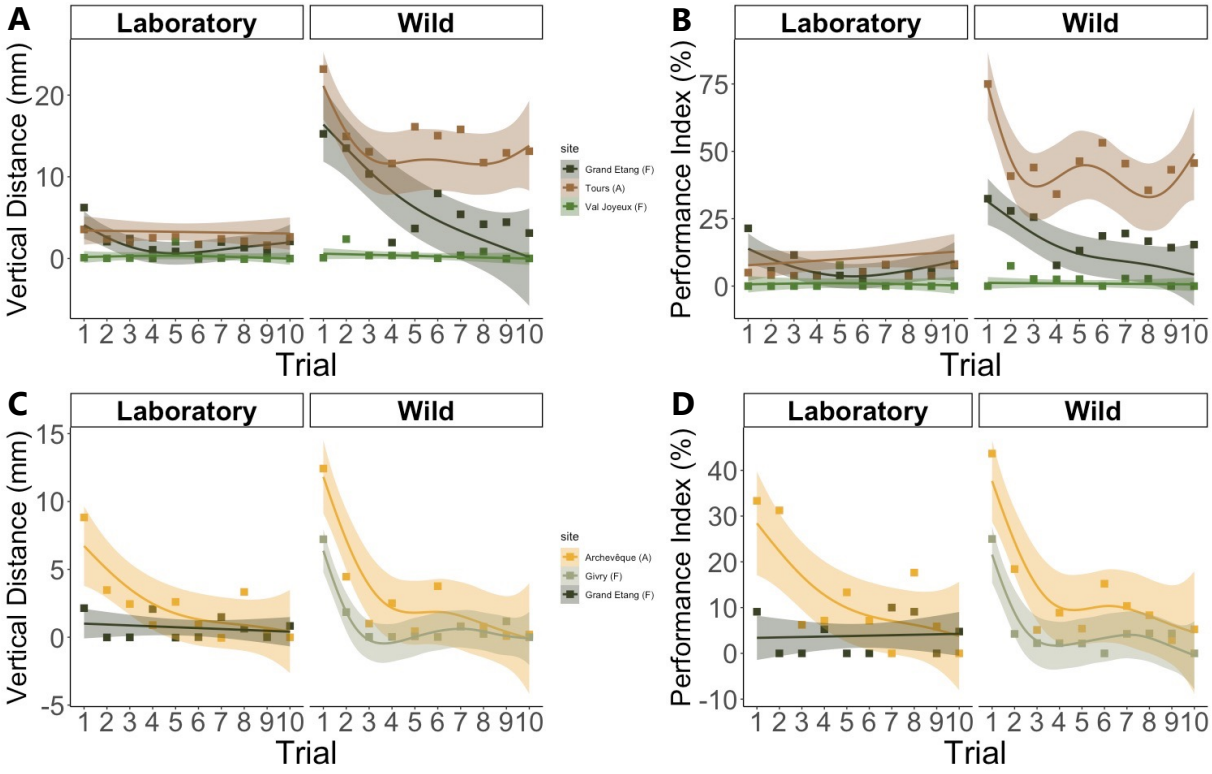

Figure 20: Courbe de la performance d'apprentissage des individus collectés sur le terrain (Chapitre 5) selon les deux variables utilisées : distance verticale en millimètres (A, C) et index de performance en pourcentage (B, D).

Ce dernier point met en avant une perspective technique réalisable. En effet, notre dispositif est assez élémentaire pour pouvoir être adapté à une étude qui se situerait directement sur le terrain. Il serait envisageable de **dimensionner un système portatif** à mettre en place à quelques mètres d'un étang afin de mesurer le comportement des larves sans avoir à les transporter ni à les maintenir en milieu artificiel. En dehors d'avantages logistiques et techniques, ce procédé nous permettrait d'avoir une approche approfondie afin de démêler les différents facteurs environnementaux qui peuvent impacter les capacités cognitives de larves moustiques, présentés dans le **Chapitre 5**. Nous avons en effet montré un fort impact du maintien des larves pendant sept jours en laboratoire, avec des performances diminuées. Pour cela, il faudrait adapter la caméra et la source de lumière à une batterie portative et modifier le système actuel pour comprendre des attaches rapides et un ajustement de l'intensité et de la hauteur du stimulus, via impression 3D par exemple. Le système devrait également être contenu dans un système clos afin de contrôler la luminosité, et réduire l'effet du vent ou d'autres stimuli (autres organismes, poussières, etc). Un

système Peltier pourrait également venir constituer le socle du dispositif afin d'ajuster la température, même si de l'eau déchlorée à température voulue pourrait être préparée au préalable. Ce système aurait l'avantage d'être facilement déployable à grande échelle et facilement reproductible via la création de notices techniques accessibles en open-source.

Enfin, nos travaux pointent du doigt des **différences inter-individuelles** à explorer. Malgré la rigueur imposée en laboratoire pour maintenir et entrainer nos individus, plusieurs facteurs peuvent modifier le comportement de nos individus. Par exemple, la performance de nourrissage et la compétition inter-individuelle peuvent modifier la vitesse de développement et l'état de satiété des individus dès les premiers stades larvaires (Mackay et al. 2023). Même avant le premier stade larvaire, la phase de quiescence de l'œuf et ses réserves énergétiques peuvent être variables, avec la larve de premier stade étant en état pharate, c'est-à-dire déjà à l'état de larve mais toujours dans l'œuf (Perez et Noriega 2013; Di Battista et al. 2022). Certains auteurs parlent de personnalité chez les larves de moustique, sous le terme de « behavioural syndrome » (Reiskind et Janairo 2018), qui correspond à un ensemble de traits comportementaux corrélés à l'échelle de la population. Les individus ont tendance à produire différents types de comportements qui se retrouvent dans différents contextes (Lucon-Xiccato et al. 2024). De plus, de nombreuses études étudient les variations individuelles chez les adultes (Carrasco et al. 2019). Il semble donc pertinent de développer des expériences qui étudient d'autres traits physiologiques et comportementaux chez les larves de moustique afin de mettre en évidence des corrélations avec le stade adulte.

La cognition des larves de moustique : comparaisons et perspectives

Ce travail de thèse s'inscrit dans l'ensemble des expériences qui étudient l'habituation chez les invertébrés. Via notre série d'expérience, nous avons validé plusieurs critères définis par Thompson et Spencer et revus par Rankin et al. (2009). Cependant, **d'autres critères sont à étudier** afin de mieux comprendre le phénomène

d'habituation chez la larve de moustique. D'abord, plusieurs séries d'entrainement pourraient être facilement mis en place à la suite, afin de mesurer les critères #3 et #10, c'est-à-dire une conservation de l'habituation entre plusieurs séries d'entrainements et une habituation à long terme. Nos résultats du **Chapitre 3** sont particulièrement opportuns à mettre en perspective avec ce type d'expérience car nous n'avons pas trouvé de mémoire à long terme via une série d'entrainement. De plus, dans les **Chapitre 2 et 5**, nous n'avons pas mis en évidence une réelle déshabituation pour les genres *Culex* et *Anopheles,* car la réponse au test chez le groupe Contrôle N°1 n'était pas significativement plus forte qu'au 10ème essai. Comme précisé par Rankin et al. (2009), d'autres méthodes peuvent être mises en place pour distinguer l'adaptation sensorielle et la fatigue de l'habituation, comme par exemple la sensibilité à la réponse spontanée en fonction de la durée de l'intervalle entre les essais (Rankin et Wicks 2000). Il serait également pertinent d'analyser la réponse spécifique à la vibration mécanique et voir dans quelles proportions les larves pourraient s'habituer à ce stimulus aversif de forte intensité qui simule un mouvement dans l'eau. Les stratégies employées par les larves pourraient être différentes entre des stimuli aériens et aquatiques, et alors qu'un stimulus aérien entraine une réponse de fuite, des stimuli aquatiques pourraient favoriser l'immobilité. Cette hypothèse pourrait également faire échos aux résultats du **Chapitre 5** et notamment à la faible activité et à la faible réponse comportementale des individus qui ont évolué dans des environnements forestiers. Ces derniers présentent des fortes concentrations d'herbiers aquatiques de type macrophytes, qui constituent des refuges pour les larves. Ainsi, en évoluant dans un milieu densément peuplé de ce type de structure, les larves pourraient avoir privilégié une stratégie où elles diminuent leur locomotion et favorisent la perception de signaux mécaniques et olfactifs à la place de stimuli visuels. Chez les crabes, les réponses à différents types de stimuli peuvent entrainer des réactions de fuite ou d'immobilité, et ces réponses dépendent des espèces considérées (Maldonado 2002; Hemmi et Tomsic 2012).

L'habituation, apprentissage non-associatif, révèle des subtilités fondamentales importantes qui permettraient d'approfondir les potentielles applications de notre dispositif. Notre travail s'est intéressé à la modalité visuelle, mais **la perception des moustiques est multisensorielle**. S'intéresser aux autres modalités ainsi qu'à l'intégration croisées des informations ouvre un fascinant champ de questions,

notamment le trade-off entre les différentes voies sensorielles lors du développement larvaire (Wheelwright et al. 2021). De plus, une autre piste d'amélioration est également accessible, en adaptant notre système à l'apprentissage associatif.

Concernant la **modalité olfactive**, de précédentes études ont mis en évidence une perception de signaux olfactifs de prédateurs ou de conspécifiques chez les larves de moustique. Par exemple, Roberts (2012, 2014) a montré que les larves des espèces *Culex quinquefasciatus, Culiseta longiareolata* et *Culex sinaiticus* modifiaient leur comportement de filtration en surface ou en profondeur si elles étaient en présence de phéromones de prédateurs (« kairomones »), c'est-à-dire de salamandres, larves de libellules ou de demoiselles, de poissons ou de nèpe (punaise aquatique). Bien que les réponses étaient différentes en fonction des espèces, les larves ont de manière générale diminué leur comportement de fourragement en profondeur et augmenté leur fourragement en surface.

Jusqu'à présent, seules trois études ont tenté de déterminer un **conditionnement olfactif** chez les larves de moustique, sous la forme d'un apprentissage sensible au degré de prédation chez les larves des espèces *Aedes triseriatus* et *Culex restans* (Kesavaraju et al. 2007; Ferrari et al. 2007, 2008). Dans chacune de ces études, les larves de moustique sont d'abord exposées à une eau avec ou sans prédateur (odeur de salamandre ou d'un prédateur moustique : *Toxorhynchites rutilus*). Dans un second temps, elles sont exposées à une eau qui a contenu des prédateurs seuls ou des prédateurs et des conspécifiques, à plusieurs concentrations nommées « *risques* ». Dans le second cas, les larves ont présenté une augmentation de leur activité ou ont passé plus de temps au repos à la surface, et cette activité a été corrélée avec la concentration de risque. Les auteurs suggèrent alors que les larves pourraient associer l'odeur d'un prédateur ou de conspécifiques à un risque de prédation, après une seule exposition, et ainsi adapter leur comportement de manière adaptative. Cependant, comme le souligne Lutz et al. (2017), on ne sait pas précisément comment les larves utilisent leurs capacités olfactives pour moduler leur comportement.

Concernant la **modalité visuelle**, il n'y a pas d'évidence dans la littérature d'un **apprentissage associatif** entre un stimulus visuel et un stimulus aversif ou appétitif chez la larve de moustique. Puisque les résultats dans la modalité olfactive sont prometteurs, et qu'un conditionnement a été montré chez les adultes, chez des espèces du même ordre, ainsi que chez des espèces qui possèdent des structures nerveuses plus petites, il semble parcimonieux de dire que les larves de moustique pourraient sans doute faire preuve de conditionnement visuel (Wolff et al. 2022). En début de thèse, nous avons mené une **étude préliminaire** afin de répéter une expérience précédemment effectuée dans notre équipe, qui consistait à associer un stimulus visuel (un contraste visuel faible, en tant que stimulus conditionnel) à un stimulus mécanique (vibration à forte intensité, en tant que stimulus inconditionnel). J'ai ainsi développé un système permettant de filmer en direct un individu, en effectuant une séquence particulière de stimulations visuelles et mécaniques, puis extrait les données de déplacement de la tête, du thorax et de l'abdomen des individus (Figure 3). Notre dispositif présentait cependant plusieurs impasses techniques et nous nous sommes redirigés vers l'habituation via le dispositif développé au **Chapitre 2**. Cependant, l'expérience accumulée lors de cette première phase, ainsi que les améliorations techniques introduites tout au long de la thèse nous permettent aujourd'hui de proposer une manière d'adapter notre dispositif au paradigme associatif développé ici (Figure 3).

L'enjeu est de créer un stimulus visuel neutre, représenté par un éclairage à intensité modérée, sur un ou plusieurs axes qui entourent les larves de moustique. Ce stimulus représenterait un contexte visuel, et serait apparié à un stimulus aversif via les moteurs vibrateurs ou le bras robotisé, représentants respectivement un prédateur aquatique ou aérien. Dans leur environnement naturel, les larves de moustique pourraient apprendre à associer ce contexte visuel de manière conditionnelle, afin d'adapter leur comportement. La démonstration d'un tel apprentissage associatif chez les larves permettrait non seulement d'ouvrir une nouvelle voie d'étude sur les capacités cognitives des larves de moustique et des mécanismes sous-jacents, mais également de développer une nouvelle méthode afin d'estimer la qualité des écosystèmes aquatiques.

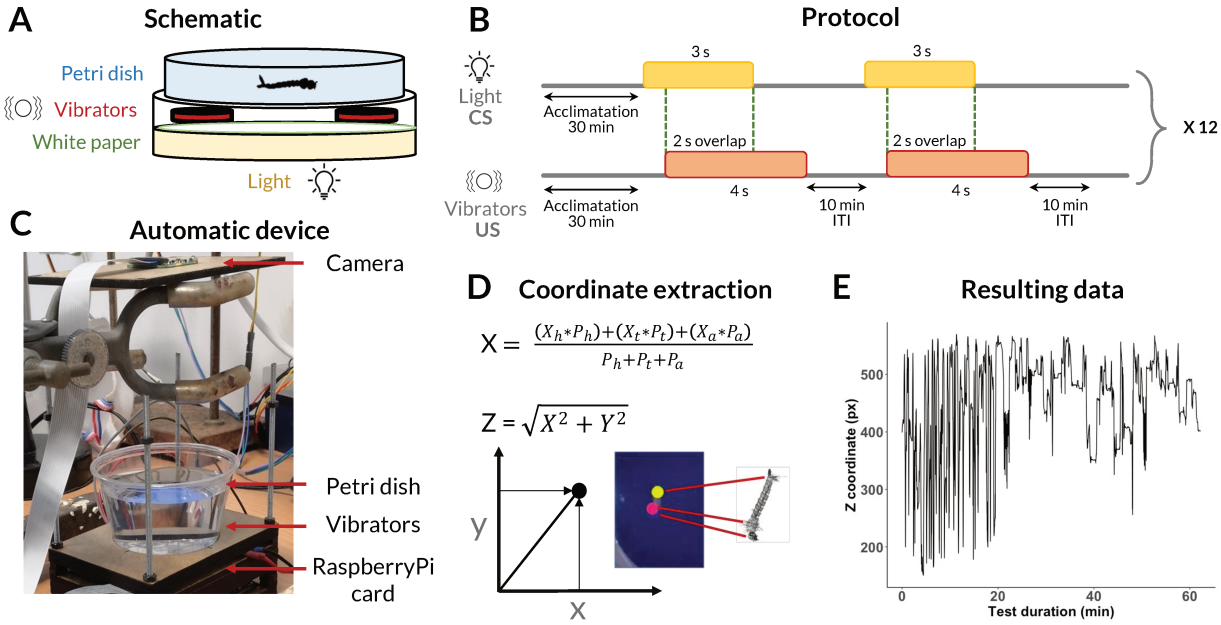

Figure 21: Expérience préliminaire : conditionnement visuel chez Aedes aegpyti. A) Schéma du dispositif : bleu : boite de Petri ; Rouge : moteurs vibrateurs ; vert : filtre papier permettant de diminuer l'intensité du contraste visuel ; Jaune : lampe. B) Protocole expérimental utilisé. Après une phase d'acclimatation, la lumière est allumée pendant 3-4 secondes, puis 1-2 secondes après, les vibrateurs sont enclenchés. 12 essais sont réalisés, espacés de 10 minutes d'intervalles. La zone en rouge est celle où l'on observe le comportement des larves. C) Dispositif automatisé : un ordinateur raspberry Pi pilote un écran situé sous la boite de Petri transparente. Il pilote aussi les moteurs situés contre la boite de Petri , et la caméra fixée au-dessus. D) Trois données sont extraites : les coordonnées en X et Y de la tête (Xh), du thorax (Xt), et de l'abdomen (Xa). A chaque coordonnée est associée un indicateur de fiabilité (Ph, Pt, Pa). La variable Z est calculée afin d'estimer le déplacement moyen des individus. E) Exemple de données de déplacement.

Remise en contexte écologique : vers l'établissement d'un indicateur biologique

Un des enjeux de ce travail de thèse est d'établir un **nouvel indicateur biologique** des écosystèmes d'eau douce en utilisant les capacités cognitives des larves de moustique. Bien que nos résultats soient satisfaisants et œuvrent vers le développement d'un tel indicateur, certains points ne sont pas évalués et restent à préciser. En effet, en reprenant la liste des douze **critères proposée par Bonada et al. (2006)**, et même si Bonada et al. utilisent des catégories d'indicateurs et non pas un exemple comme le nôtre, une première estimation de cet indicateur est possible (Figure 4). De nombreux points restent à explorer et à développer, notamment la dimension d'identification et de discrimination des fonctions écologiques et des types de

perturbations anthropiques (critères III, V, X, XI). En effet, il est encore impossible à présent d'identifier la présence de polluants ainsi que leurs qualités via notre dispositif. Bien que nous ayons mis en évidence des effets de l'environnement sur la cognition des larves, nous ne pouvons pas à ce jour séparer les effets abiotiques et biotiques de la présence de polluants, ni de leurs multiples interactions. Bien qu'à priori, de nombreuses relations entre concentrations de polluants et réponses comportementales soient linéaires, il se peut qu'il y ait des seuils de réponses et d'autres formes de réponses (critère XII, Bonada et al. 2006).

	Critère évalué	
Justification	(I) Dérivé de concepts théoriques en écologie	+
	(II) Prédictif a priori	+
	(III) Possibilité d'évaluer les fonctions écologiques	?
	(IV) Possibilité d'identifier les perturbations anthropiques globales	+
	(V) Possibilité de distinguer différents types de perturbations anthropiques spécifiques	?
Implémentation	(VI) Faibles coûts d'échantillonnage et de tri et d'expérimentation en laboratoire	+
	(VII) Protocole d'échantillonnage simple	+
	(VIII) Faibles coûts d'identification des taxons (aucun spécialiste en taxonomie n'est requis)	+
Performance	(IX) Applicabilité à grande échelle (à travers les écorégions ou les provinces biogéographiques	+
	(X) Indication fiable des changements dans l'impact humain global	?
	(XI) Indication fiable des changements dans les différents types d'impact humain	?
	(XII) Indication de l'impact humain sur l'échelle linéaire	?
	Total des critères en concordance	7
	Total des critères en non - concordance	0
	Total des critères qui restent à explorer	5

Figure 22: résumé de la position de l'utilisation des capacités cognitives des larves de moustique confrontée au 12 critères proposés par Bonada et al. (2006).

Toutefois, notre modèle permet de valider les trois critères liés à l'implémentation en proposant un système à faible coût, un travail de terrain accessible sans matériel spécifique et une identification simple au moins jusqu'au genre (Critères VI, VII, VIII ; voir **Chapitre 2 et 5**, section « *Methods* »). Ce système est également applicable à grande échelle dans le sens où les moustiques se retrouvent à l'état larvaire sur chaque continent, en spéculant que les larves de chaque espèce devraient pouvoir faire preuve d'apprentissage. A minima, les genres *Aedes, Culex* et *Anopheles* sont

répandus mondialement et peuvent former une base concernant leurs capacités d'habituation (Critère IX). L'effet de polluants sur le comportement des macro-invertébrés aquatiques est bien documenté, et malgré le peu de données expérimentales concernant les études sur les capacités d'apprentissage, plusieurs concepts écologiques peuvent être établis qui permettent de valider le critère I. Par exemple, le concept de niche écologique permet de prédire des conditions physiologiques sous-optimales d'un organisme vivant dans un milieu donné. De plus, il est possible de prédire l'effet direct de polluants sur les larves de moustique en utilisant les données mécanistiques d'études analogues et nos données concernant l'effet de trois polluants (critère II). Enfin, en montrant que des polluants d'origine anthropique pouvaient modifier les capacités cognitives des larves, nous pouvons proposer cet indicateur pour identifier un impact global anthropique (critère IV).

Ce travail de thèse s'inscrit dans le projet régional COMPORTATE, dont l'objectif principal est de caractériser les conditions des écosystèmes d'eau douce en région Centre-Val de Loire. Cette région est formée de champs ouverts, de forêts et de zones humides, unifiée par le Val de Loire. Elle possède un axe ligérien très dynamique, industrialisé et avec une activité agricole diverse, qui impacte la riche biodiversité, notamment des organismes d'eau douce. Par exemple, la région possède un Plan national d'actions en faveur des odonates de 2020 à 2030 (https://odonates.pnaopie.fr/).

En combinant notre approche de biologie comportementale avec des **approches de mesures physiques et chimiques de l'eau et des habitats**, nous sommes en capacité de mieux qualifier ces écosystèmes (Oertel et Salánki 2003). Pour l'instant, les résultats présentés dans le **Chapitre 5** ne nous ont pas permis de procéder à ces corrélations. En effet, nous avons sélectionné des étangs à priori qui présentent des structures plus ou moins anthropisées. Nous avons prédit que l'anthropisation aurait un effet délétère sur le comportement des larves de moustique, et estimé les différences de prédation, de structure de l'habitat et de pollution (Chapitre 5, Table 1). Alors que **l'effet de prédation** a fait l'objet d'une étude de terrain approfondie grâce à l'association naturaliste *Caudalis*, la **structure de l'habitat** et le **niveau de pollution** sont pour

l'instant sous forme d'estimations. Grâce à l'expertise de deux laboratoires de chimie organique et analytique, collaborateurs de ce projet régional, nous pourrons avoir une approche corrélative plus complète. Durant trois ans, ces laboratoires ont collecté des échantillons d'eau, de matière organique, et ont placé des loggers de température afin de déterminer 1) les conditions physico-chimiques des étangs et 2) la présence de substances polluantes, en particulier d'herbicides et de médicaments. Les doses « field-realistic » utilisées dans le **Chapitre 4** proviennent notamment de relevés effectués par ces laboratoires en amont de notre travail. Le but est donc d'avoir une réelle détermination de l'état de ces étangs, directe par l'analyse classique de l'eau, et indirecte via notre étude sur la biologie comportementale des larves, ainsi que via l'estimation de la diversité des odonates. La mise en commun de l'ensemble de nos données permettra une étude corrélationnelle de l'état des milieux ainsi qu'une analyse critique de cette approche multidisciplinaire.

Par ailleurs, certains paramètres de chimie organique ont déjà été corrélés avec la présence et le développement des larves, mais pas de leur comportement (Rydzanicz et al. 2016; Avramov et al. 2023). L'intégration de ces approches permet d'avoir une vision détaillée de la situation en Centre-Val de Loire, ainsi que d'ouvrir des perspectives fondamentales concernant la biologie, l'écologie et la cognition des moustiques. Par exemple, ces approches permettent d'ouvrir des perspectives concernant les potentielles adaptations ou résistances des moustiques aux polluants et aux insecticides (Dada et al. 2019).

Afin d'aller plus loin dans la validation de ce type d'indicateur biologique, il est primordial de **mieux comprendre le rôle des polluants** sur le comportement des larves de moustique. Dans notre étude, nous nous sommes restreints au dernier stade larvaire mais d'autres études indiquent des **changements de comportement entre les différents stades** (Thomas 1949; Tuno et al. 2004; Reiskind et Janairo 2018). Ainsi, de nouvelles expériences pourraient comparer le rôle de polluants à différents stades de développement, en comprenant également les performances cognitives à l'âge adulte afin d'avoir une idée de l'impact des polluants sur les cycles de vie complets des moustiques. Afin d'aller plus loin, des études transgénérationnelles pourraient étudier l'effet de polluants sur les générations filles issues de populations soumises à des

polluants, ce qui serait une étape cruciale pour mieux discerner l'effet population dans l'analyse d'individus collectés en milieux naturels (**Chapitre 5**). Par exemple, une étude récente a montré l'effet du cuivre sur le comportement anti-prédation chez les larves de *Culex pipiens*, sur deux générations. L'exposition au cuivre a diminué la vitesse et la distance parcourue avec un effet plus fort sur la deuxième génération (Amer et al. 2022). Ainsi, reproduire les moustiques en laboratoire permettrait d'évaluer les effets délétères des polluants à long terme.

Les moustiques présentent des **rythmes, notamment circadiens** (Clopton 1979), ainsi que des variations saisonnières de leur activité et développement (Rydzanicz et al. 2016). Plusieurs études ont mis en évidence la présence d'une horloge circadienne endogène et les mécanismes moléculaires qui la régulent, ainsi que leur rôle primordial dans la régulation des comportements chez les adultes (Gentile et al. 2013). Cette horloge a également un rôle sur la physiologie des larves, comme le montre une étude où le temps de développement larvaire a été augmenté lorsque des gènes impliqués dans la régulation des cycles ont été désactivés (Shetty et al. 2022). Chez d'autres insectes, les rythmes circadiens modulent également les capacités d'apprentissage. Par exemple, la punaise *Rhodnius prolixus* présente de bonnes capacités d'apprentissage durant la nuit, mais pas la journée (Vinauger et Lazzari 2015). La drosophile *Drosophila melanogaster* présente également une modulation circadienne de la mémoire à court terme (Lyons et Roman 2009). Dans chacune de nos études, la période d'entrainement a été effectuée durant l'après-midi, afin de s'astreindre d'un potentiel effet circadien. De plus, les larves sont contraintes à se déplacer dans un volume d'eau fini et sont potentiellement soumises en permanence à des prédateurs ou d'autres stimuli. Cependant, nous pouvons nous demander si les capacités d'apprentissage, de mémorisation et les activités de locomotion pourraient être modulées par un rythme circadien. Notre système semble effectif afin de répondre à ces questions.

Une troisième perspective est celle d'examiner plus précisément la **structure de l'habitat** où sont présentes les larves de moustique. Par exemple, Rejmánková et al. (2006) ont examiné l'occurrence de structures d'habitats, de nutriments et du choix de

sélection par les moustiques femelles dans une étude corrélative et une expérience de terrain et de laboratoire. D'après leurs résultats, ils suggèrent que les perturbations anthropiques, que ce soit par l'ajout de substances dans l'eau ou de modification des sols, peuvent perturber la chaine trophique des écosystèmes d'eau douce. Les larves de moustique pourraient, en particulier, profiter de l'eutrophisation de ces milieux qui changerait la structure de leur habitat. L'eutrophisation augmenterait la présence de macrophytes denses qui deviendraient de meilleurs refuges pour les larves et des sites idéals de pontes pour les femelles (Rejmánková et al. 2006). D'autres études ont montré l'influence de la structure de l'habitat directement sur les capacités cognitives d'invertébrés. Par exemple, une élégante étude menée par Pull et al. (2022) a analysé la mémoire à court terme chez le bourdon *Bombus terrestris* durant deux ans. Au printemps, lorsque les ressources sont nombreuses, les bourdons doivent se rappeler sur quelles fleurs ils ont butiné au sein d'un même patch. Ainsi, leur mémoire à court terme, testée dans un labyrinthe à quatre bras, indique une bonne performance, ce qui n'est pas le cas durant l'été, lors des périodes de disette où les ressources sont rares. Des modifications de la réponse comportementale de fuite dans le cadre de l'habituation ont également été démontrées chez le crabe *Neohelice granulata* en fonction de la disponibilité de refuges à proximité (Hemmi et Tomsic 2015).

Par ailleurs, la présence de larves de moustique est corrélée avec la **présence de prédateurs**, qui jouent un rôle plus ou moins prévalent. Les prédateurs peuvent agir sur l'abondance des larves (Priyadarshana et Slade 2023), sur leur comportement anti-prédation (Evans et al. 2019) et dans certains cas peuvent influencer leurs capacités d'apprentissage (Vila Pouca et al. 2021). En partant du postulat que les larves de moustique présentent une forte résistance aux perturbations, l'observation d'altérations pourrait indiquer des altérations plus importantes chez d'autres organismes sympatriques plus sensibles. En particulier, ces effets pourraient se retrouver chez des prédateurs aquatiques comme aériens, comme le montre une étude de Akhtar et al. (2021) concernant les effets de métaux lourds bioaccumulés par les larves *Aedes aegypti*. De plus, des espèces controphiques (c'est-à-dire, qui partagent le même niveau trophique que les larves de moustique) pourraient également entrer en jeu

dans la répartition et l'exposition des larves de moustique à des polluants (Blaustein et Chase 2007).

Enfin, tous nos entrainements se font de manière individuelle. Or, bien que les moustiques soient des espèces solitaires, les larves vivent très proches les unes des autres et peuvent percevoir des signaux olfactifs de conspécifiques (Kesavaraju et al. 2007). Ainsi, des perspectives multiples s'ouvrent quant aux **possibles formes d'interaction entre les individus**. Nous pourrions nous demander si une forme de communication existe entre deux individus et si elle pourrait modifier leur comportement et leur faculté d'apprentissage. En mettant deux larves dans la même enceinte, soumise à un stimulus visuel aversif, avec un individu qui est habitué et l'autre non, nous pourrions prédire que l'individu naïf pourrait s'habituer plus vite. L'individu entrainé pourrait également se déshabituer en réponse au comportement de fuite de son partenaire. Ce contexte social pourrait modifier les capacités cognitives des individus comme cela a été montré chez d'autres insectes (Lihoreau et al. 2019). Dans un milieu naturel, les larves pourraient bénéficier de la perception de leurs congénères afin d'adapter leur comportement à des stimuli plus ou moins saillants. A l'image des règles comportementales simples qui dirigent le comportement des individus formant un banc de poisson, les larves de moustique pourraient également posséder des seuils de réponse différents et leur position vis-à-vis de conspécifiques ainsi que leur état physiologique pourraient entrainer des réponses comportementales variées.

Ainsi, il semble **trop tôt** pour parler de l'utilisation des capacités cognitives des larves de moustique comme **véritable indicateur biologique**. Dans cette thèse, nous apportons une première **preuve de concept** de cette idée, avec des résultats importants en écotoxicologie couplés à un système robuste. Notre solution s'inscrit parmi d'autres, notamment celles proposées par l'entreprise ViewPoint qui utilise des gammares afin d'avoir un suivi en temps réel de la présence de polluants via des modifications comportementales (Ruck et al. 2023; Soose et al. 2023). Notre système a la particularité de mesurer des **effets sous-létaux précis** directement liés aux **capacités cognitives des larves de moustique**. Cependant, ces résultats doivent être **répliqués** et

approfondis afin de tenter de cocher tous les critères permettant le développement de ce type d'indicateur.

Conclusion générale

Ce travail de thèse propose une preuve de concept de l'utilisation de la cognition des larves de moustique afin de nous renseigner sur l'état des écosystèmes d'eau douce. Notre outil est à l'état de prototype mais s'il est développé, pourrait permettre à la fois d'explorer les capacités cognitives des espèces aux plus **forts enjeux sanitaires** et qui sont le plus en **expansion**, et à la fois de **sensibiliser** pour la **conservation des écosystèmes les plus en danger**.

Notre travail s'inscrit également dans un **projet régional** multidisciplinaire : celui de caractériser l'état de santé des écosystèmes d'eau douce de la région Centre-Val de Loire par des approches complémentaires. Parmi celles-ci, notre approche est indirecte, en prenant le rôle fondamental du comportement comme adaptation biologique, et en choisissant de mesurer les performances cognitives des larves de moustique.

Notre travail s'inscrit aussi dans une approche **éco-éthologique**, c'est-à-dire via l'étude du comportement en lien avec le contexte écologique. Dans le cadre de ce projet régional, nous proposons une **approche transversale** qui permet de répondre à des problématiques fondamentales et appliquées à plusieurs échelles : écosystémique, via notamment les possibles interactions entre moustiques, odonates, et différents habitats ; populationnelle, via la caractérisation des organismes de plusieurs étangs ; individuelle, via ces travaux de thèse ; et moléculaire, via les analyses de chimie organique et analytique en cours.

Enfin, notre travail s'inscrit dans une **perspective de conservation**. Comme beaucoup d'auteurs le suggèrent, la biologie expérimentale peut concrètement servir des projets à vocation conservatoire, et vice versa (Buchholz 2007; Greggor et al. 2014;

Roth et Krochmal 2015; MacDonald et Ritvo 2016; Pritchard et al. 2016; Morand-Ferron et al. 2016; Dominoni et al. 2020). En effet, ce mutualisme constitue une manière efficace de comprendre les **interactions multitrophiques** au sein et entre les écosystèmes, avec la possibilité d'intégrer des facteurs de **changements globaux** et de **pollution anthropique**. Elle permet de simuler des **situations réalistes**, par exemple via l'intégration **d'effets cocktails** de polluants sur la cognition des larves de moustique. De plus, cette approche permet d'explorer des **propriétés fondamentales** chez les organismes étudiés, notamment des capacités cognitives de haut niveau chez les insectes et leurs mini cerveaux (Heyes 2012). Cette approche est également traduisible au grand public par un **axe de sensibilisation** aux problématiques liées aux écosystèmes. Par exemple, notre projet régional comprend des activités de **vulgarisation** et la création d'une **exposition** au Museum de la ville de Tours sur les écosystèmes d'eau douce. Ce point souligne notre approche expérimentale par une volonté de **partager des connaissances** et notre approche de terrain par une **dynamique de questionnement des ressources naturelles** qui nous entourent. Enfin, le rayonnement de notre approche a pour but d'aller conseiller décideurs, politiques et gestionnaires de terrain. Grâce à notre démarche intégrative, nous pouvons interpeller ces responsables avec des images compréhensibles, concrètes, fiables et justifiées (Oertel et Salánki 2003). Ce public, qui peut être étranger à la démarche scientifique, pourrait être plus sensible à des images comme « l'intelligence de la larve de moustique », ou « s'imaginer dans la tête d'une libellule ». L'emploi de ces espèces porte-drapeaux pourrait permettre plus facilement de prendre conscience des enjeux de conservation des écosystèmes d'eau douce.

Il est néanmoins important de garder une note de **réalisme** concernant la situation saisissante de la **dégradation massive** des écosystèmes, ainsi que la science de la conservation comme étant une « science de la crise » qui nécessite des réponses rapides, efficaces et précises (Stirling et Burgman 2021). Certains auteurs soulignent les incohérences entre les décisions économiques et politiques liées à la conservation des écosystèmes et insistent sur la nécessité de l'engagement des chercheurs au détriment de leur neutralité axiologique (Ellison 2016; Oberdorff 2022; Lynch et al. 2023). Toutefois, la plupart des dommages sont d'origine anthropique et pourraient donc, si l'on décide

d'y consacrer du temps et de l'énergie, être réduits ou éliminés (Klein et al. 2017; van der Sluijs et al. 2021).

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Annexes

Journal of Insect Physiology 149 (2023) 104535

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Assessing learning in mosquito larvae using video-tracking

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ARTICLE INFO

Keywords:
Habituation
Non-associative learning
Aedes
Culex
Anopheles

ABSTRACT

Mosquito larvae display a stereotyped escape response when they rest attached to the water surface. It consists in detaching from the surface and diving, to return to the surface after a brief time. It has been shown that this response can be evoked several times, by repeatedly presenting a moving shadow. Diving triggered by a potential danger revealed as a simple bioassay for investigating behavioural responses in mosquito larvae, in particular their ability to learn. In the present work, we describe an automated system, based on video-tracking individuals, and extracting quantitative data of their movements. We validated our system, by reinvestigating the habituation response of larvae of *Aedes aegypti* reared in the laboratory, and providing original data on field-collected larvae of genera *Culex* and *Anopheles*. Habituation could be demonstrated to occur in all the species, even though it was not possible to induce dishabituation in *Culex* and *Anopheles* mosquitoes. In addition to non-associative learning, we characterised motor activity in the studied species, thanks to the possibility offered by the tracking system to extract multiple variables. The here-described system and algorithms can be easily adapted to multiple experimental situations and variables of interest.

1. Introduction

Adapting individual behaviour on the basis of the own experience (i.e. learning) and remember past experiences (i.e. memory) is crucial for an animal to survive and to make decisions (Evans et al., 2019).

Habituation is a particular form of non-associative learning (Thomas, 1949; Leftwich, 1954) which consists in no longer reacting to stimuli that trigger behavioural response in naïve animals and turned out to be innocuous (See Rankin et al., 2009 for review). For example, when a moving object casts its shadow over the water surface, mosquito larvae dive escaping from a potential danger (Holmes, 1911). After several passages of an innocuous shadow, larvae stop responding, even though they still detect it and they are able to dive. The individuals do not perceive the stimulus as a potential danger anymore, i.e. the larvae become habituated to its presence (Baglan et al., 2017).

Habituation protocols in mosquito larvae have revealed to be reliable bioassays, not only for testing cognitive abilities of these insects (e.g. Baglan et al., 2017; Pientrantuono et al., 2021), but also as a proxy for evaluating the impact on living creatures of chemical pollutants in water (Baglan et al., 2018). In a typical experiment, an observer records whether or not individual larvae move during the controlled passage of a moving shadow, attributing a score of 0 or 1. The shadow is presented at regular intervals, until the insects stop responding. Specific tests follow, in order to assess whether the behavioural change is either due to learning or to other physiological processes, such as sensory adaptation or motor fatigue (for a review, see Rankin et al., 2009).

In this work, we present an original system allowing the automated quantification of different components of the response of mosquito larvae to a potential danger. Our tracking software allows accurately measuring individual response, and calculating different metrics associated with diverse components of the behavioural response, minimising experimental biases.

The system allows training and testing several individuals in parallel in a single session, saving time, increasing the number of replicates, and obtaining accurate quantitative data on different behavioural variables. Training parameters as intensity and duration of the stimulus, inter-trial interval, interval between training and test can be precisely adjusted by the experimenter. We started by testing and validating our system and the experimental protocol for habituation experiments in a reference species (i.e. *Aedes aegypti*) and then we compared the responses among laboratory and field-collected mosquito larvae of other species.

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https://doi.org/10.1016/j.jinsphys.2023.104535
Received 9 May 2023; Received in revised form 29 June 2023; Accepted 2 July 2023
Available online 5 July 2023

2. Material and methods

2.1. Animals

Aedes aegypti (Bora strain) were obtained from eggs provided by the INFRAVEC2 European project and reared at VECTOPOLE-IRD (Montpellier, France). The eggs were placed in small plastic containers filled with dechlorinated tap water and fed *ad libitum* with shrimp food (JBL Novo Prawn, Neuhofen, Germany). The larvae were maintained in a climate-controlled room at 25 °C ± 2 °C, under 12 h:12 h light:dark illumination regime (lights on at 8:00).

Culex and *Anopheles* larvae were collected in two natural habitats located in the department of Indre et Loire, France. The first site was a 10-ha basin (*Étang de l' Archevêque*) located in the Loire Valley (47°31′N, 0°51′E), and the second was a pond situated in an urban garden in the city of Tours (47°23′N, 0°41′E). At each site, captures were carried out over a sampling area of approximately 1 m^2 by scooping the surface with a 1-litre round recipient (O'Malley, 1995), until at least 100 individuals were collected. Sampled individuals were kept in water from their natural habitat during the journey back to the laboratory and then gently transferred to 750 ml polypropylene plastic containers and reared similarly as *Ae. aegypti* larvae. Before experiments, individuals were kept undisturbed between 24 h and 48 h under laboratory conditions (the same for all species). Fourth-instar larvae were used in all the experiments.

All animals were collected, reared and manipulated according to ethics regulations applied in the European Union.

2.2. Identification

For *Culex* and *Anopheles*, the morphological identification of individuals was performed under a stereomicroscope, with the aid of the MosKeyTool database (Gunay et al. 2018). An initial identification was conducted after the experiment to assess the genus at larval stage. Individuals were kept until emergence, then adults were identified a second time to determine sex and confirm genus, using the same key.

We found at least two species of *Culex* using the Moskeytool database: *Culex pipiens* and *Culex territans*. We were able to distinguish these two species by comparing their siphon index (total length/diameter at the base) and the arrangement of their siphon setae. For the genus *Anopheles*, it was much more difficult to determine the species, but it is most likely that the individuals belonged to the *Anopheles maculipennis* complex. The Moskeytool database did not go further than this complex and we could not find any visible differences between the *Anopheles* individuals. We also identified the genus *Culiseta*, but we excluded these larvae, because they were rarely found in our samples.

2.3. Experimental setup

The experimental apparatus (Fig. 1), consisted of two light sources, a camera, and a servo mechanism, which controlled the projection of the shadow of a square cardboard (*shadow*) above twelve 1.5 ml spectrophotometer plastic cuvettes (Z187992-1PAK, Sigma-Aldrich, Germany) where the larvae had been individually placed. One light source consisted of two LED panels (30 cm × 30 cm), located above the cuvettes. The second light source was an infrared backlight (880 nm) placed behind the cuvettes. In front of the cuvettes, a camera (acA 1300 – 60gc, Basler, Germany) equipped with a high-pass infrared filter (RG 850 Filter – 40.5 mm, Heliopan, US) recorded the experiments.

In order to exclude unwanted vibrations, cuvettes stood on a 2 cm-thick polystyrene plate resting over acoustic foam. The lateral faces of each cuvette were covered with opaque white tape, in order to avoid mutual visual influence. A water-filled cuvette without larvae was placed at the left of the 1st cuvette and another one at the right of the 10th cuvette, to minimise any effect of cuvette position.

Two stimuli of different modality could be presented. The first (visual) was the shadow projected by a black cardboard square (16 cm side) attached to a wooden stick and fixed to a servomotor controlled by an Arduino Uno board (https://www.arduino.cc). During a stimulation, the stick turned 100° and returned back to the resting position (Fig. 1). The second stimulus (mechanical) was the vibration produced by a set of 4 identical vibrators (3.3 V, 100 mA; 11000 rpm; 10 mm diameter; 2.7 mm height, Radio Spares, France), controlled by the same Arduino Uno board. Two vibrators were placed on the outer side of the left end and

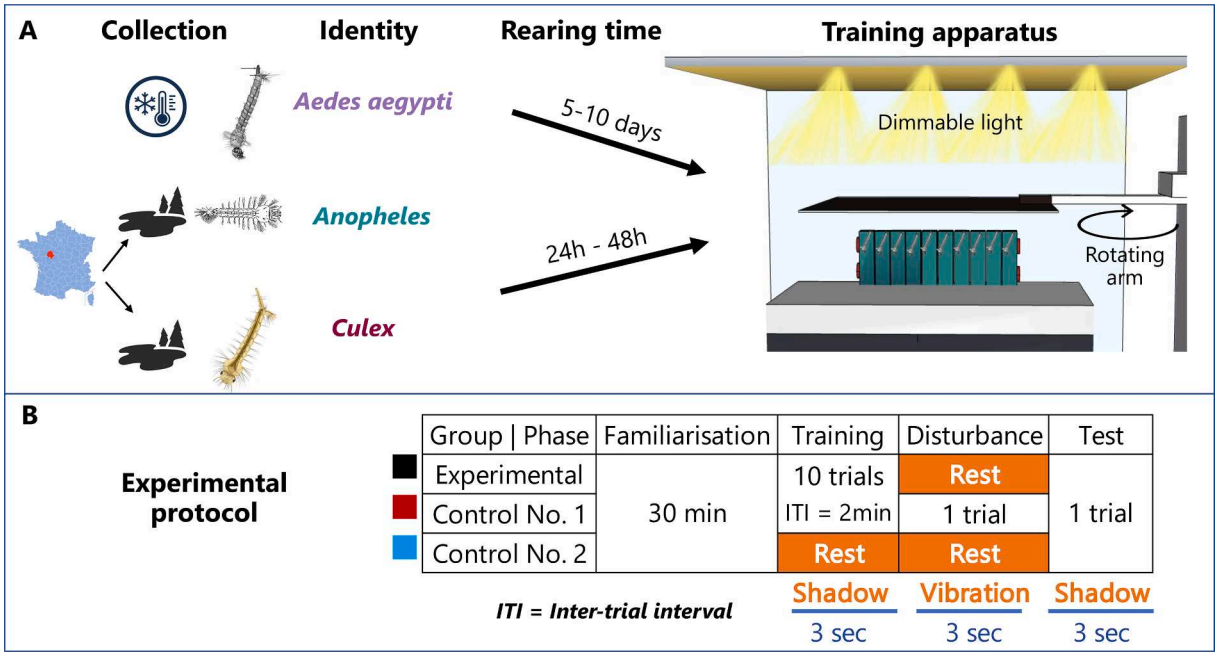

Fig. 1. Experimental protocol. A) We collected *Culex* and *Anopheles* larvae in two ponds located in Indre-et-Loire, Région Centre-Val de Loire, whilst *Aedes aegypti* larvae were reared in the laboratory. We trained individuals of the 4th larval stage using an automated device. B) We quantified the responses of three groups: 1) trained and not disturbed (Experimental); 2) trained and disturbed (*Control No. 1*); 3) untrained (*Control No.* 2).

the other two at the right end, i.e., on the side of the unoccupied cuvettes. The Arduino board was remotely controlled by a computer, which was also connected to the camera.

Preliminary tests revealed differences in the responsiveness of the larvae belonging to the different species. For this reason, a series of experiences were run in order to establish the appropriate parameters for testing each species.

For *Ae. aegypti* light intensity was set at 900 μW.cm^{-2} ± 100 μW.cm^{-2} (International Light Technology radiometer). The distance between the top of the cuvettes and the rotating arm was established in 5 ± 0.2 cm and the stimulus duration fixed at 3 s at an angular velocity of 0.067°/ms.

For *Culex* and *Anopheles* larvae, we increased the light intensity to 1500 μW.cm^{-2} ± 100 μW.cm^{-2} and placed the card closer to the top of the cuvette (0.3 ± 0.1 cm). We also increased the arm rotation to 7.5°/ms and added 1.5 s of delay in the stimulus position, to keep the total stimulus duration at 3 s. The goal was ensuring that most of the larvae would react.

2.4. Experimental conditions

All experiments were performed in a room kept at the rearing temperature. Larvae were carefully removed from the rearing container and placed individually in the cuvettes filled with dechlorinated tap water.

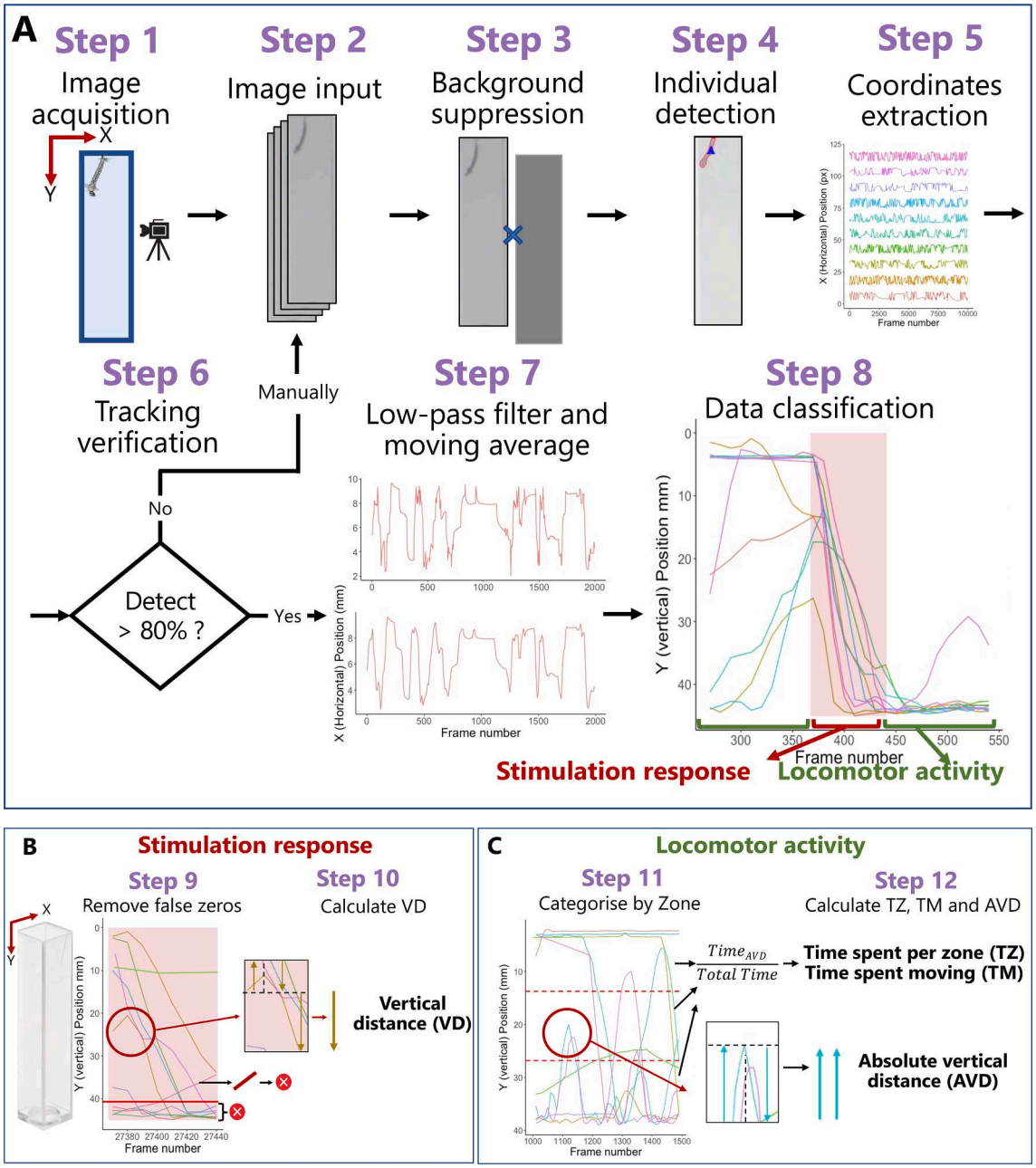

Fig. 2. Recording and quantifying individual behaviour. A) Flowchart of data acquisition and treatment. B) Response to visual stimulus (SR) was analysed using two metrics. Performance Index (PI) was binary and calculated following the trajectory direction of each individual for each trial. Vertical distance (VD) was quantitative and calculated as the relative sum of the distance travelled in the vertical direction. C) Locomotor activity (LA) was analysed using three metrics. Time spent per zone (TZ) was a proportion of time spend in one of the 3 zones delimited. Time spent moving (TM) was a proportion of time where the Absolute vertical distance was above a threshold of 1 mm/sec. Absolute vertical distance (AVD) was quantitative and calculated as the absolute sum of the distance travelled in the vertical direction.

Larvae were left undisturbed during 30 min for familiarisation before starting the experiment. Under these conditions, we established one experimental group and two control groups. The procedure consisted of three phases: *training or rest*, *disturbance or rest* and *test* (Fig. 1).

The *Experimental* group was set to assess the decrease in response induced by the repeated presentation of the visual stimulus (*training*). Larvae were confronted with 10 passages of the shadow (i.e. *trials*), spaced by inter-trial intervals (ITI) of 2 min. After the 10th presentation of the stimulus, larvae remained undisturbed during 4 min, before the final presentation of the shadow; i.e., the *test* phase.

Control No. 1 (disturbance) was set to assess dishabituation (Rankin et al., 2009). Larvae were exposed to 10 stimuli, similarly to the experimental group (*training*). Two minutes after the 10th stimulus, a vibration was applied (*disturbance*). This disturbance was followed by 2 min ITI and the final presentation of the shadow (*test*).

Control No. 2 (untrained), after familiarisation, larvae remained confined in the cuvettes during 22 min without receiving any stimulation. Subsequently, the visual stimulus was presented to the larvae only in the *test* phase.

After the end of each experiment, larvae were gently removed from the cuvettes and individually kept in identified Petri dishes (3-cm diameter) during 24 h. Those that emerged as adults during this time could have been pharate pupae during the experiment and consequently excluded from the analyses.

2.5. Video analyses

Each experiment was recorded and two sets of videos (resolution 640x480 px, 25 fps) were produced (Fig. 2, step 1). The first one consisted of sequences of the last 5 min of familiarisation. The second set consisted of 26 min videos of the three phases of each experiment (i.e., *training*, *disturbance* and *test*). The videos were analysed using a modified version of the image-based freeware Tracktor (Sridhar et al., 2019).

The tracking software was based on a contour identification algorithm relying on the contrast between the larvae and the background. (Fig. 2, step 2 and 3). During the video analysis, the position and contour area of each larva were measured while keeping identity (Fig. 2, step 4). At the end of the video analysis measurements were exported to a.*csv* file.

The tracking results were analysed using R version 4.1.1 (2021-08-10) (https://cran.r-project.org/). Horizontal coordinates were used to verify that larvae identities were respected (each larva detected inside a given cuvette, Fig. 2, step 5). To check the performance of the tracking, the detection rate was calculated by taking the maximum frame length available on the video and multiplying it by the number of individuals. This rate was compared to the actual number of frames identified by the tracking software and we ensured that at least 80% of the data present (Fig. 2, step 6). The vertical position data were smoothed using the *rollmean* function in the *zoo* package (Zeileis and Grothendieck, 2005) with a 10-frame window (Fig. 2, step 7).

2.6. Data classification and analysis

From each dataset and each trial, we defined and extracted the 3 s trial period as the duration of the stimulus appearance over the individuals (Fig. 2, step 8). Therefore, we could extract 8 successive positions for each individual and for each trial that were classified as Stimulation Response dataset (Fig. 2).

No vertical displacement could be observed in the larvae that were at the bottom of the cuvette at the beginning of a trial. Therefore, we excluded the response of individuals whose vertical position at the start of a trial was higher than 9/10th of the cuvette length (i.e. close to the bottom) (Fig. 2, step 9).

Vertical distance (VD) was the response variable and corresponded to the escape response, starting from 0 at the top of the cuvette, and increasing, when larvae dived along the water column (Fig. 2, step 10). We also defined a binary criterion as:

Therefore, we calculated the proportion of individuals (i.e., Performance Index, PI) that dived enough to be considered responding.

For *Culex* and *Anopheles* species, some individuals rested completely motionless during one trial. For this trial, the value given for their displacement was therefore counted as 0 mm for VD and for PI. When one individual was completely immobile during the acclimation and the training period, it was removed from the database (8.5% for *Anopheles*, 0% for others). A total of 246 individuals were retained for the analysis (Table 1).

As individual positions were recorded throughout the whole experiments, we also extracted data during the 9 inter-trial intervals (ITIs) and analysed the locomotor activity during these periods (Fig. 2). We first calculated the Absolute Vertical Distance (AVD) travelled by individuals by summing the AVD for all ITIs per individual during the training session (Fig. 2, step 11). We also ranked data by ITI and compared the AVD per ITI for each species. The AVD was then averaged per second and calculated for each individual to compare the individual average speed during the ITIs. The maximum speed of each individual was also compared in the same way.

We divided the cuvette in three equal zones (top, middle, bottom, Fig. 2, step 12) and calculated the time spent per zone. We used these zones to develop another metric corresponding to the diving events. If an individual crossed two successive zones on the way in and out, we considered this to be a diving event. To analyse if an individual was moving or not based on a dichotomous rule, we also confronted the individual AVD to a threshold of 1 mm per second and classified the resulting data in Time spent moving (Fig. 2, step 12). Prior to any training, individuals were recorded for 30 min during the familiarisation period. To highlight the effect of the stimulation on individual activity, we analysed the last 5 min of familiarisation and compared them to the ITI periods. Finally, using contour tracking data, we were able to compare the maximum individual surface detected by the tracking between species, i.e., the area representing each individual in pixel.

3. Statistical analyses

3.1. Data classification and filtering

For the three species, we verified whether responses to stimulation were trial-specific (i.e. increased or decreased) by applying a Chi-square goodness of fit test. The rationale behind this verification was to exclude that larvae could have changed their position before the release of the stimulus over the course of the training, then biasing the output of the filtering.

3.2. Power analysis

Using the *"simr"* package in R (Green and MacLeod, 2016), we performed a power analysis to confirm the power of our sample size. For the Vertical Distance variable and for each species, we used the function *powerSim*. For 1000 simulations and alpha = 0.05, the power was 95.90% CI [94.48, 97.04] for *Aedes aegypti*, 89.50% [87.43, 91.33] for *Culex* and 98.70% [97.79, 99.31] for *Anopheles*.

3.3. Comparison across species

For the three mosquitoes, we used a Generalised Additive Model to explore different response curves for each variable and their effect on the generalised cross-validation unbiased risk estimator (GCV-UBRE) (Zuur et al., 2009). We defined models of increasing complexity and different smoothing functions and compared them using the GCV-UBRE of the *mgcv* package (Wood, 2017).

To compare locomotor activity between species, we used linear mix-effects models. These models were used for the comparison of AVD (m), average speed (mm/s) and maximum speed (mm/s), time spent per zone

Table 1
Summary of the filtering steps. For each species, 22 to 30 individuals were trained. When the individual's position was close to the bottom, the response to the trial was removed, accounting for a total of 13.9% of trials removed. The trajectory of an individual moving upwards during a stimulation was rare, representing only 3.4% of trials.

	Aedes aegypti				*Culex*				*Anopheles*				All
	Experimental	Control n°1	Control n°2	Total	Experimental	Control n°1	Control n°2	Total	Experimental	Control n°1	Control n°2	Total	Total
Individuals trained	30	30	30	90	26	29	27	82	22	28	24	74	246
Trials per individuals	360	360	30	750	310	347	27	684	191	313	24	528	1962
Trials filtered by position	274	268	24	566	271	312	27	610	189	301	23	513	1689
% Trials removed	23.9%	25.6%	20.0%	24.5%	12.6%	10.1%	0.0%	10.8%	1.0%	3.8%	4.2%	2.8%	13.9%
Trials filtered by going up	268	263	24	555	261	290	26	577	184	293	23	500	1632
% Trials removed	2.2%	1.9%	0.0%	1.9%	3.7%	7.1%	3.7%	5.4%	2.6%	2.7%	0.0%	2.5%	3.4%
Total % Trials removed	25.6%	26.9%	20.0%	26.0%	15.8%	16.4%	3.7%	15.6%	3.7%	6.4%	4.2%	5.3%	16.8%

(%) and time spent moving (%), number of diving event and maximum surface (mm^2). We chose species as a fixed factor and individual identity as a random factor. Post-hoc comparisons were analysed using the *emmeans* function from the *emmeans* package (Russell, 2021).

3.4. Learning performance

To assess the learning performances of the different groups of larvae, we compared Vertical Distance and Performance Index. VD was evaluated by means of a linear mix-effects model and PI of a GLMER with a logit link and a binomial distribution, with trial number and group as fixed factors, and individual identity as random factor to account for repeated measurements. The group factor served to evaluate eventual effects due to contexts across groups of larvae trained in a similar way. As the interaction between trial and group was never significant, we dropped the interaction from the model.

3.5. Test phase

Test responses were analysed by running a linear mix-effects model for VD and a GLMER for PI with a logit link and a binomial distribution. VD and PI were chosen as the response variables; group as fixed factor, and individual identity as random factor.

3.6. Dishabituation

To assess dishabituation, we compared VD and PI at the tenth trial with the response at the *disturbance* and at the *test* phase. VD was compared by using a linear mix-effects model and PI by means of GLMER, similarly as in the section Learning performance.

3.7. Dataset and analysis code repository

The version of the tracking software used to characterise individual behaviour and the R code used to analyse data and display the figures were made available online at: https://github.com/martindessart/Tracking-system.

4. Results

4.1. Identification

Larvae and adults could be identified at the genus level. For *Anopheles,* we were able to evaluate the sex of 25 individuals out of the 74 total individuals trained, of which 13 were identified as females and 12 as males. For *Culex,* 40 individuals were identified as females and 24 as males.

4.2. Data classification and filtering

At 25 frames per second, a 22-minutes recording corresponded to a total of 33 000 frames. Our tracking algorithm, adapted from the open source software Tracktor (Sridhar et al., 2019) had a tracking time of<20 min. For comparison, the zebrafish video from ToxTrac software (Rodriguez et al., 2018), with a resolution of 32 frames per second and 15 000 frames, had a tracking time of 9 min 43 s using Tracktor software (Sridhar et al., 2019). Regarding accuracy, the total percentage of detection rate was 92.54% for 22 records. The highest detection rate was 99.97% and the lowest 84.84%. For each experiment, we performed a calibration by zooming in on the cuvettes with our camera and applying the function "Automatic image adjustment" from Basler Pylon5 software (https://baslerweb.com). Provided that each cuvette was physically separated from the others, a handmade function on R software using the total horizontal distance (*x*-coordinate) divided by the number of individuals allowed us to identify all individuals for all videos. Finally, for each recording, we manually selected the square outline of the 10

cuvettes. Then, for each species, we took the mean of the maximum distance in pixels for each record and converted the pixel unit to millimetres. The mean distance was: Mean 401.4 px, SD = ±9.01 px.

Our tracking system was able to discriminate individuals based on their vertical position at the start of each stimulation. When an individual was above a threshold of 9/10th of the total length of the cuvette, the filtering step (Fig. 2, step 9) eliminated an average of 13.9% of the trials for all species (Table 1). This percentage depended on the species, with 24.5% for *Ae. aegypti,* 10.8% for *Culex* and 3.0% for *Anopheles* Furthermore, the binary criterion detected and eliminated an additional 3.3% of responses to trials where individual VD was greater than 1/5th of the maximum VD. This was more pronounced for *Culex* (5.4% of removed trials) than for *Anopheles* (2.4%) and *Ae. aegypti* (1.9%). The overall process resulted in 1632 stimulation responses for 246 individuals. To determine whether the trials deleted in these two successive steps were trial-specific, we confronted this hypothesis using a Chi-square goodness of fit test. The deleted trials were not specific to a trial number for the three species: *Ae. aegypti*: $X^2 = 10.81$, df = 11, P = 0.459; *Anopheles:*$X^2 = 8.11$, df = 11, P = 0.703; *Culex*: $X^2 = 15.59$, df = 11, P = 0.157.

The performance of our automated system was compared to human visual characterisation. Three different experimenters scored manually the behavioural response of *Aedes aegypti* by looking at the videos. Overall, the confidence intervals were larger for the visual classification (Experimenter #1: 95% CI [0.94, 1.06], Experimenter #2: 95% CI [0.92, 1.08], Experimenter #3: 95% CI [0.93, 1.07]) than for the automated approach (95% CI [0.95, 1.04]). In addition, there were significant differences in scores between the three experimenters. The percentage of unequally scored data between Experimenter #1 and Experimenter #2 was 34.4%; Experimenter #1 and Experimenter #3 was 26.2% and Experimenter #2 and Experimenter #3 was 32.5%. Overall, the percentage similarity between the three experimenters was 53.4%. So, visual categorisation revealed less precise than the automated one.

4.3. General comparison across species

The response of the larvae decreased with the consecutive passage of the shadow. In other words, the escape response was less intense over the course of the ten trials, both for PI and VD (Fig. 3A, B). Concerning PI, a difference in the number of responsive larvae (Escape response = 1) was observed across species, being *Ae. aegypti* the most responsive and *Anopheles* the least. All three decreased across trials at similar rates, curves running parallel at different levels.

To describe the variation in VD, the best smoothing function was the P-spline, as it is based on equally spaced knots (Wood, 2017). Plotting the mean distance (mm) against the number of trials for the three species revealed different responses (Fig. 3B). *Ae. aegypti* responded strongly to the stimulus (Mean = 22.69, SEM = ±0.63), *Culex* was weaker than *Ae. aegypti* (Mean = 16.09, SEM = ±1.11) and *Anopheles* responded the least (Mean = 6.50, SEM = ±0.1) (Fig. 3A, B). *Anopheles* also decreased their response more steeply than *Ae. aegypti* and *Culex* (Fig. 3A, B).

Concerning spontaneous locomotor activity, *Ae. aegypti* and *Culex* moved significantly more than *Anopheles* (P < 0.0001 in both cases) but did not differ from each other (P = 0.758); see supplementary Fig. S1A. Regarding within-trial differences, the three species did not show significant differences among trials (*Ae. aegypti*: df = 8, P = 0.990, *Culex:* df = 8, P = 0.999, *Anopheles*: df = 8, P = 0.100); see supplementary Fig. S1B. Regarding the average speed, while *Ae. aegypti* and *Culex* were significantly faster than *Anopheles* (P < 0.0001 in both cases, Supplementary Fig. 2A), the latter reached higher maximum speed than *Ae. aegypti* and *Culex* (P < 0.0001 in both cases). *Culex* had a higher maximum speed than *Ae. aegypti* (P < 0.0001) but was not faster (P = 0.488); see supplementary Fig. S2A, B. Similarly, *Anopheles* spent little time moving (ca. 11% of the time), while *Ae. aegypti* was very active (ca. 80% of the time) and *Culex* was moderately active (ca. 47% of the time); see supplementary Fig. S3B). While *Anopheles* spent more than 75% of its time near the surface, *Culex* spent more than 25% in the middle and at the bottom zone of the cuvette and *Ae. aegypti* spent more time at the bottom zone; see supplementary Fig. S3A. The difference in activity was maintained when comparing the number of diving event with *Ae. aegypti* and *Culex* diving more than *Anopheles* (both P < 0.0001), but there was no difference between *Ae. aegypti* and *Culex* (P = 0.690): see supplementary Fig. S4A. On average, *Ae. aegypti* and *Anopheles* images had similar surface area (in pixels) (P = 0.438) and were larger than *Culex* (both P < 0.0001); supplementary Fig. S4B).

Finally, movement comparisons between familiarisation and ITI for *Ae. aegypti* showed no difference in average speed (P = 0.834); see supplementary Fig. S5A), but a significant difference in maximum speed (P < 0.0001); supplementary Fig. S5B). The comparison of time spent moving show little difference (P = 0.046); supplementary Fig. S5D.

4.4. Training phase

Learning performance was assessed by comparing individual responses between the 1st and the 10th trials (Fig. 3). For the three species, these comparisons rendered significant differences, evincing a decrease

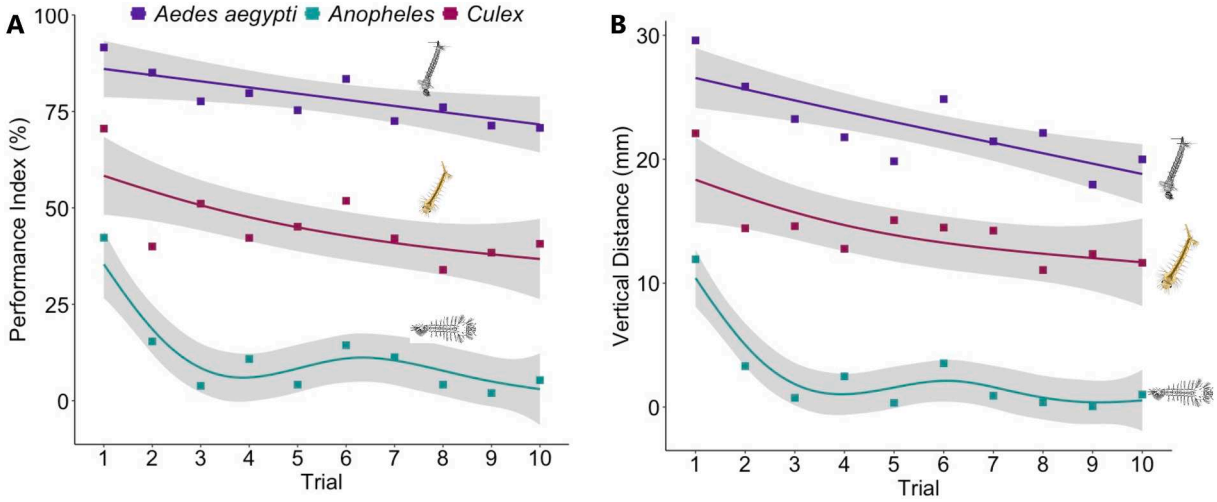

Fig. 3. Behavioural response over the course of the training phase. A) Performance Index of individuals responding to the visual stimulus in each *training* trial. B) Vertical distance in millimetres travelled by individuals responding to the visual stimulus from the 1st till the 10th *training* trial. Smoothing lines indicate the best fitted GAM model. Grey shades indicate 95% confidence interval. Points indicate mean values.

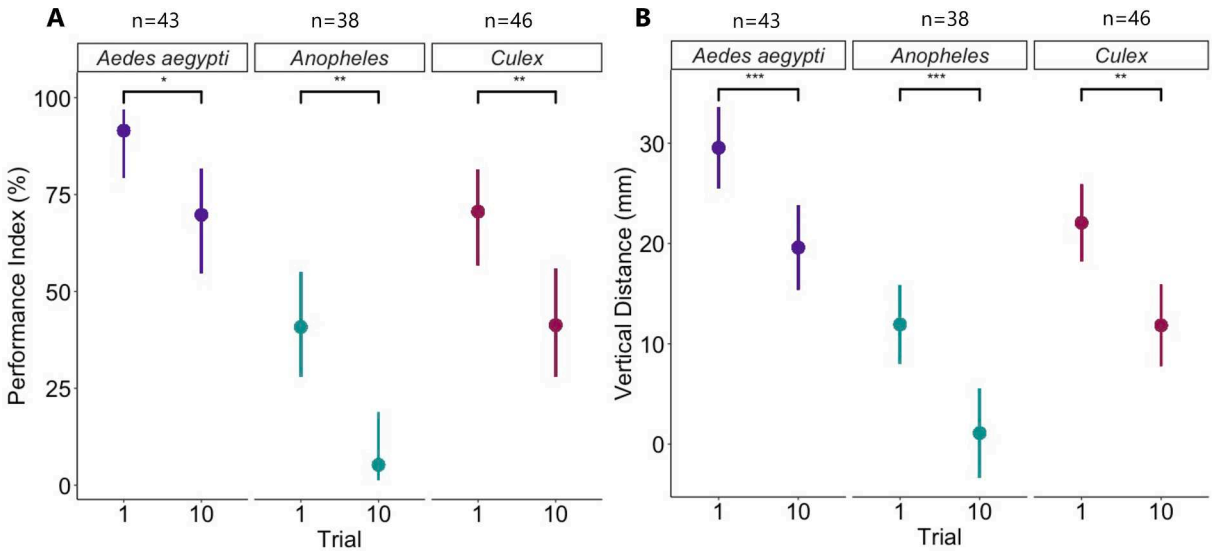

Fig. 4. Learning performance. A) Performance Index of individuals responding to the visual stimulus in the 1st trial and in the 10th trial. B) Vertical distance travelled by individuals responding to the visual stimulus in the 1st trial and in the 10th trial of the *training* phase. Points indicate mean values and bars indicate +- 95% confidence intervals. NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.001.

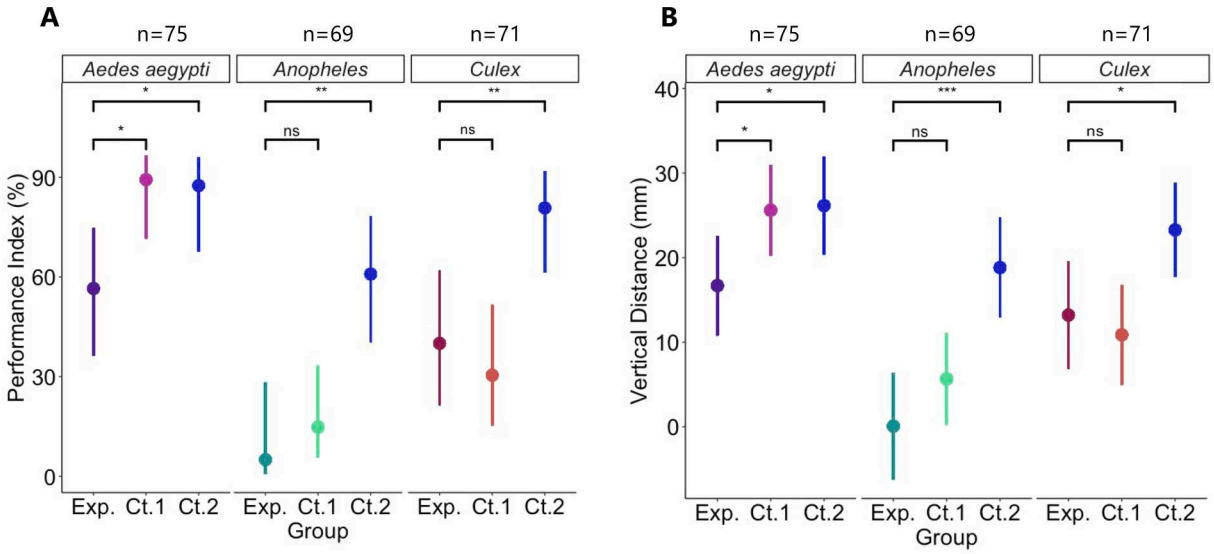

Fig. 5. Test phase. A) Performance Index of individuals responding to the visual stimulus during the *test* phase (i.e. after the *training* phase). B) Vertical distance travelled by individuals responding to the visual stimulus during the *test* phase. Dark purple, dark green and dark red indicate *experimental* group (Exp.) for each species. Light purple, light green and light red indicate *Control No. 1* (Ct.1) for each species. Blue indicates *Control No. 2* (Ct.2) for each species. Points indicate mean values and bars indicate +- 95% confidence intervals. NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in responsiveness (Fig. 4). The Performance index was higher at the 1st than at the 10th trial (*Ae. aegypti:* $X^2 = 5.93$, df = 1, P = 0.015; *Anopheles:* $X^2 = 9.17$, df = 1, P < 0.01; *Culex:* $X^2 = 8.01$, df = 1, P < 0.01) (Fig. 4A). Vertical distance (mm) was also higher at the 1st than at the 10th trial (*Ae. aegypti:* $X^2 = 13.786$, df = 1, P < 0.001; *Anopheles:* $X^2 = 17.957$, df = 1, P < 0.001; *Culex:* $X^2 = 10.472$, df = 1, P < 0.01) (Fig. 4B).

4.5. Habituation assessment

For each species, we compared the response at the *test* trial on PI and VD between *Experimental* group, *Control No. 1* and *Control No. 2* (Figs. 5, 6). For *Ae. aegypti*, the response level of the *Experimental* group was significantly lower than in *Control No. 1* (PI: 95% CI [0.40, 4.02], P = 0.016; VD: $t_{70} = 2.53$, P = 0.014) and in *Control No. 2* (PI: 95% CI [0.21, 3.92], P = 0.029; VD: $t_{70} = 2.66$, P = 0.010, Fig. 6). For *Culex* and *Anopheles*, the response of the *Experimental* group was significantly lower than that of *Control No. 2* (*Culex* PI: 95% CI [0.48, 3.32], P = 0.009; VD: $t_{64} = 2.07$, P = 0.042; *Anopheles* PI: 95% CI [1.17, 6.34], P = 0.004; VD: $t_{66} = 4.00$, P < 0.001) but not relative to *Control No. 1* (*Culex* PI: 95% CI [-1.75, 0.86], P = 0.507; $t_{63} = 0.47$, P = 0.643; *Anopheles* PI: 95% CI [-1.23, 3.49], P = 0.347; VD: $t_{66} = 1.38$, P = 0.173), concerning both PI and VD (Fig. 5).

By comparing individual response between the 10th *training* trial and the *test* phase (i.e, after the disturbance), we looked for evidence of dishabituation to occur. Both, the PI and VD showed contrasted performance for dishabituation across species. *Ae. aegypti* was the only out of the three mosquitoes analysed to show a reversal of the habituation

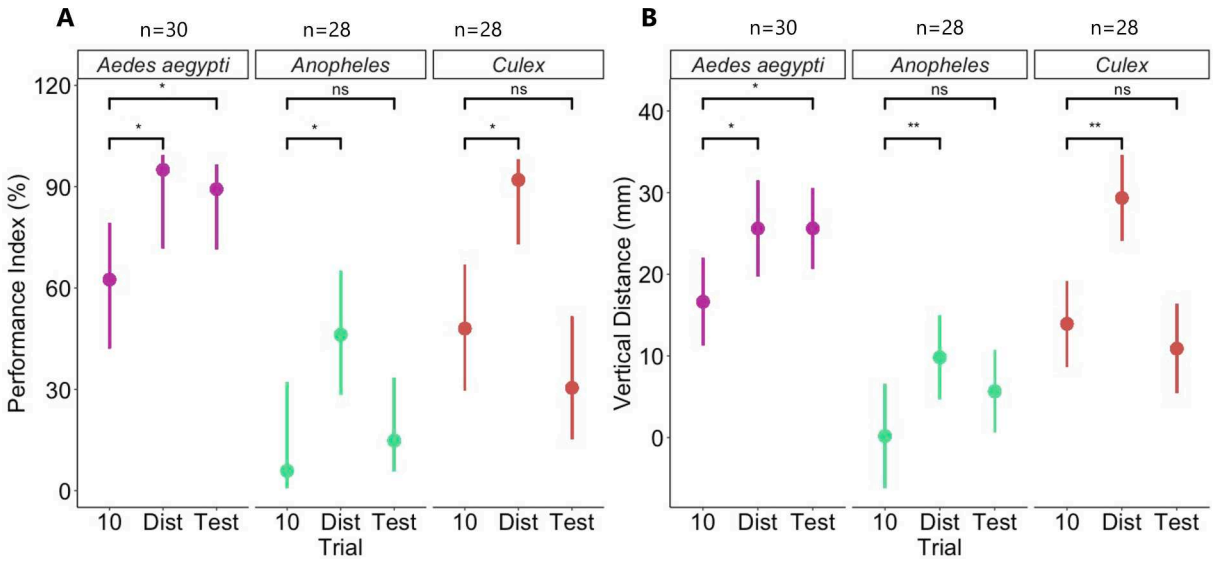

Fig. 6. Dishabituation. A) Performance Index of individuals responding to the visual stimulus in the 10th trial, the *disturbance* phase and in the *test* phase). B) Vertical distance travelled by individuals responding to the visual stimulus in the 10th trial, n the disturbance phase and in the *test* phase. Dist = disturbance, i.e. the mechanical stimulation between the two trials. Points indicate mean values and bars indicate +- 95% confidence intervals. NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.001.

induced by training (Fig. 6). *Culex* and *Anopheles* remained not responsive even after the mechanical disturbance. Yet, all three species evinced an increase in responsiveness when the mechanical disturbance was applied (*Ae. aegypti* PI: 95% CI [0.26, 4.61], P = 0.028; VD: $t_{67} = 2.42$, P = 0.018; *Culex* PI: 95% CI [0.81, 4.27], P = 0.004; VD: $t_{68} = 3.72$, P < 0.001; *Anopheles:* PI: 95% CI [0.46, 4.78], P = 0.018; VD: $t_{65} = 2.72$, P = 0.008; Fig. 6). *Ae. aegypti* showed a significant difference between the 10th trial and the *test* phase for both Performance Index and vertical distance (PI: 95% CI [0.15, 3.06], P = 0.030; VD: $t_{67} = 2.64$, P = 0.010). In contrast, the PI and VD of *Culex* and *Anopheles* were not significantly different between the 10th trial and the *test* phase (*Culex* PI: 95% CI [0.26, 4.61], P = 0.028; VD: $t_{68} = -0.75$, P = 0.456; *Anopheles:* PI: 95% CI [-1.96, 0.45], P = 0.221; VD: $t_{65} = 1.49$, P = 0.141; Fig. 6).

5. Discussion

The goal of the present work was to introduce a novel automated system for evaluating the diving response of mosquito larvae, to validate it with insects belonging to different species and having different origins. We showed that the tracking method and the algorithms developed revealed as useful, rendering accurate sets of data and assuring replicability. Automated tracking methods facilitate behavioural quantitative analyses (e.g. Panadeiro et al., 2021). In our work, different behavioural variables could be quantitatively analysed, allowing comparing performances across mosquito species.

We have been able to investigate habituation in mosquito larvae. As expected (Baglan et al., 2017), *Ae. aegypti* larvae were able to habituate to a visual stimulus initially perceived as dangerous, and control groups allowed to distinguish habituation from fatigue and sensory adaptation (Thompson, 2009). For *Culex* and *Anopheles,* a significant decrease in the escape response occurred and convergent evidence supported the occurance of habituation in these mosquitoes also. On the one hand, the nature of the stimulus (a passing shadow) and the time elapsed between the last training essay and the test (i.e. several minutes), make sensory adaptation unlikely. On the other hand, the intense response triggered by mechanical disturbance allows excluding motor fatigue.

We calculated two main variables, the Performance Index (PI) and the Vertical Distance (VD) travelled by the larvae. PI was conceived as an easy-to-use binary variable to determine the proportion of individual response to the visual stimulus. This variable is analogous to observations that would have been made by a human experimenter, the major difference was the classification process. By setting a threshold to classify individuals as moving or not on the basis of their relative movement, we avoided classifying brief spontaneous movements or erratic behaviour as positive responses to the visual stimulus. We also ensured that the response interval was constant over the training (i.e. similar interval for each trial). This step was crucial especially for very active species such as *Ae. aegypti* (Jackson, 1953; Lutz et al., 2020). A characteristic of the PI is that the threshold was defined in advance, as a minimum intensity of movement for the individual to be considered as responding. In addition, a filter was applied to eliminate 'false zeros' in our zero-inflated model (Zuur et al., 2009), i.e. when individuals could not respond due to their position being at the bottom of the cuvette during the stimulation. Finally, the automated filtering and classification steps provided a robust way to keep constant the selection process over time (i.e. avoiding inter- and intra-observer variability). Thus, quantifying the response of mosquito larvae was based on objective replicable criteria instead of relying on subjective appreciation.

The Vertical Distance (VD) variable was designed to quantify the intensity of the escape response. Upon successive occurrence of the same stimulus, the intensity of a behavioural response may vary or even be completely inhibited (Evans et al., 2019). Here, VD refers to the biological escape response of mosquito larvae, which occurs primarily in the vertical direction, as described by Clements (1999).

Individual displacement was also evaluated in order to quantify spontaneous activity, using the variable Absolute Vertical Distance (AVD), i.e. the total distance travelled during all the ITI periods. Understanding the kinematics of mosquito behaviour using VD or AVD has other advantages. For instance, it allows the interpretation of movement data in a specific context by discriminating between resting period and activity, the direction of displacement, gliding motion, wriggling bouts counts, number of diving events, time spend per area, foraging behaviours, etc (Chandrasegaran et al., 2018; Lutz et al., 2020).

All the three Culicidae studied are part of the neuston (i.e. organisms associated to the water surface, either above or underneath) and, at the same time, they differ in their behaviour. *Ae. aegypti* was the most active during training and the most sensitive to the visual stimulus while *Anopheles* was the least responsive and spontaneously active, and *Culex* was in-between.

Overall, our mosquitoes significantly decreased their response

during the training phase. This variation in their responsiveness to a visual stimulus is the result of a trade-off between avoiding predation, maintaining oxygen levels and conserving energy reserves for adult emergence (Awasthi et al., 2015; Baglan et al 2017; Pientrantuono et al., 2021).

All individuals in *Control No. 1* group (i.e., *disturbance*) strongly responded to the mechanical stimulation. While our visual stimulus simulated a flying predator (Tomsic et al. 2009), the mechanical disturbance could illustrate the sudden movement of waves caused by an aquatic predator (e.g. dragonfly larvae, fish, certain mosquito larvae), and could explain the intense response to vibration of the larvae (Clements, 1999).

Finally, we found a significant difference in dishabituation in larvae, as has been the case in crabs inhabiting different habitats (see review by Tomsic et al., 2009). Yet, the lack of response at the test phase in *Control No. 1* raises the question on potential differences in learning and memory abilities across species.

In summary, we present here an automated tracking system, which revealed to be reliable, accurate and time-saving, for investigating habituation in mosquito larvae. This learning paradigm proved to be an adequate approach for studying a variety of biological questions related to mosquito cognitive abilities (Baglan et al., 2017, Pientrantuono et al., 2021, this paper) as well as the neurological impact of pollutants (Baglan et al., 2018). Other questions which could be addressed using a similar approach range from basic neurobiological mechanisms underlying, for instance memory consolidation and persistence, to ecological problems, as the impact of environmental conditions on cognition.

CRediT authorship contribution statement

Martin Dessart: Data curation, Formal analysis, Investigation, Methodology, Validation, Software, Visualization, Writing – original draft, Writing – review & editing. **Miguel Piñeirúa:** Data curation, Formal analysis, Methodology, Resources, Software, Software, Validation, Writing – original draft, Writing – review & editing. **Claudio R. Lazzari:** Supervision, Validation, Writing – original draft, Writing – review & editing. **Fernando J. Guerrieri:** Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Financial support for this work was granted by Region Centre-Val de Loire, France APR IR 2020 COMPORTATE. M. Dessart is a PhD student at the University of Tours, financed by APR IR 2020 COMPORTATE. COMPORTATE is supported by Réseau MiDi - Milieux et Diversité, Pole DREAM - French Water Team, Zone Atelier de la Loire – LTER France Loire, Réseau ENTOMOCENTRE, Département d'Indre-et-Loire -Direction de l'Attractivité des Territoires (France).

We thank Joël Meunier for material provision and fruitful discussion, David Carrasco for his invaluable advice on statistics, Carole Delavenay for support and the Doctoral School "Santé, Sciences Biologiques, Chimie du Vivant" for guidance and support. The authors express their gratitude to both anonymous reviewers for their constructive criticism and suggestions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2023.104535.

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Journal of Insect Physiology 155 (2024) 104650

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Habituation leads to short but not long term memory formation in mosquito larvae

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A B S T R A C T

In animals, memory allows to remember important locations and conserve energy by not responding to irrelevant stimuli. However, memory formation and maintenance are metabolically costly, making it worthwhile to understand the mechanisms underlying different types of memory and their adaptive value. In this study, we investigated the memory persistence of *Aedes aegypti* mosquito larvae, after habituation to a visual stimulus. We used an automated tracking system for quantifying the response of mosquito larvae to the passage of a shadow, simulating an approaching predator. First, we compared different retention times, from 4 min to 24 h, and found that mosquito larvae only exhibited memory capabilities less than 3 h after training. Secondly, we investigated the role of inter-trial intervals in memory formation. In contrast to other aquatic invertebrates, mosquito larvae showed no long-term memory even at long inter-trial intervals (i.e., 5 min and 10 min). Our results are discussed in relation to the ecological constraints.

1. Introduction

To retain information, or memory, is a crucially adaptive cognitive ability in animals (Menzel, 1999). The adaptive value of memory is related to the ability to make quick and accurate decisions when faced with a situation similar to one previously experienced (Menzel and Benjamin, 2013). Memory allows animals to avoid harmful situations, to remember important locations or specific information, and to avoid energy loss by not responding to irrelevant stimuli; in other words, memory contributes to overall fitness (Couto et al., 2023). At the same time, memory formation and maintenance have different costs (Niven and Laughlin, 2008). As the brain is metabolically expensive, the resources allocated to encode, consolidate, and access information generate important expenditures (Kandel, 2001). Different types of memory coexist, defined by their duration and the physiological processes involved in their development. They end up being adaptative or not depending on the context. For instance, in stable environments, where the probability of encountering a certain situation again is high, it may be adaptative to invest in long-term memory. In a rapidly changing environment, however, it may be better to prioritise short-term memory (Pull et al., 2022).

The properties and the physiological mechanisms underlying the different types of memory have been studied in many invertebrate models, notably in the fruit fly *Drosophila melanogaster* (Tully et al., 1994) and the honey bee *Apis mellifera* (Menzel 2001a). In addition, habituation to visual stimuli and memory has been well characterised in the mudflat crab *Neohelice granulata* (Tomsic and Silva, 2023). These experiments provided insights about the ecological relevance of memory duration according to the habitat. In a study by Tomsic et al. (1993), the authors compared the habituation of two related semi-terrestrial crabs that occupy different habitats, *Neohelice granulata* and *Pachygrapsus marmoratus*. By analysing the influence of diverse parameters on visual habituation performances (e.g., individual size, number of trials), the authors showed that habituation is species-dependent and that contextual cues are memorised differently. Tomsic et al. (1993) concluded that ecology played a major role in the origin of these differences. Indeed, *Neohelice granulata* crabs live in self-dug burrows, closed to the mud substrate and surrounded by conspecifics and halophyte vegetation. On the other hand, *Pachygrapsus marmoratus* live on rocky outcrops, close to the sea and without vegetation. So, a shadow passing over *Neohelice* crabs would induces stronger and longer habituation because it represents an ambiguous signal (e.g., grass undulation), whereas for *Pachygrapsus* crabs, the probability of being an actual flying predator would be higher in their environment which is poor in objects passing overhead (Tomsic et al., 1993), resulting in a weak habituation response in the latter.

A key parameter for habituation and the mesic mark it can generate, is the inter-trial interval (Giurfa et al., 2009). Short inter-trial intervals (e.g., from few seconds to few minutes) are more likely to reinforce short-term memory, which relies on neural facilitation (i.e., increase in synaptic strength) and reversible changes (Hemmi and Tomsic, 2012), but not long retention. In contrast, long inter-trial intervals will lead to

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https://doi.org/10.1016/j.jinsphys.2024.104650
Received 16 November 2023; Received in revised form 21 March 2024; Accepted 15 May 2024
Available online 20 May 2024

the formation of long-term memory, which depends on the activation of specific genes leading to new protein synthesis and structural changes in neural circuits (Tomsic et al., 1996; reviewed in Margulies et al. 2005 in Drosophila). In between, intermediate inter-trial intervals produce intermediate memory, which involves synaptic consolidation through the activation of specific kinases (e.g., cAMP-dependent protein kinase PKA) and early gene expression (Tomsic and Romano, 2013). While the duration of inter-trial intervals has been empirically tested, these types of memory have also been described in several taxa (Izquierdo et al., 1998; Menzel 2001b; Tully et al., 1994).

In this work, we investigated the ability to develop memory after learning in an aquatic insect, the mosquito larva (*Aedes aegypti*). Mosquito larvae spend most of their time hanging from the water surface. When a stimulus is perceived as a potential danger, larvae dive (Clements, 1999). If the stimulus turns out to be innocuous upon repeated occurrences, larvae no longer respond to further stimulation due to habituation, a form of non-associative learning, potentially forming a mnesic trace (Baglan et al., 2017; Dessart et al., 2023).

Although much attention has been paid to cognition in adult mosquitoes, this is the first study to investigate the memory of mosquito larvae. In freshwater ecosystems, mosquito larvae are part of the neuston (i.e., organisms living at the water surface). They are therefore surrounded by unpredictable aquatic and aerial predators such as dragonfly larvae or water striders (for review see: Vinogradov et al., 2022). In this type of environment, a shadow repeatedly casting over the water surface in a short period of time is likely to be projected by the same object, whereas a shadow projected over the water hours later could be produced by a different moving body. In this situation, we could expect that mosquito larvae stop to respond to the repetition of an aversive stimulation in the short term, while resetting their responsiveness in the long term, i.e., not to remember, would be a more adaptive strategy.

In addition to very-well studied aquatic invertebrates such as the sea hare *Aplysia californica* (Glanzman, 2009) or the crab *Neohelice granulata* (Tomsic et al., 2017) which exhibit remarkable forms of long-term memory, other freshwater organisms also showed consistent long-term memories, as for example crayfish *Procambarus cubensis* up to 24 h (Abramson et al., 2005), great pond snails *Lymnaea stagnalis* up to 3 days (Lukowiak et al., 2003), and water fleas *Daphnia sp*. up to 6 days (Ringelberg and Gool, 1995). Since long-term memory has been demonstrated in several aquatic species, the possibility of long-term memory in mosquito larvae cannot be ruled out without experimental evidence.

On the one hand, the highly unpredictable environment could prioritise the formation of a short-term memory in mosquito larvae. On the other hand, other organisms from similar environments show robust long-term memory. To distinguish between these two hypotheses, we conducted a series of experiments with *A. aegypti* mosquito larvae to investigate (1) how long mosquito larvae could retain information after habituation, and (2) whether the duration of inter-trial intervals would play any role in memory formation.

2. Material and methods

2.1. Animals

A. aegypti eggs (Bora strain) were provided by the INFRAVEC2 project of MIVEGEC-IRD (Montpellier, France). The eggs were reared in a climate-controlled room at 25 °C ± 2 °C and under 12 h:12 h light: dark illumination regime (lights on at 8:00). The larvae were maintained in small plastic containers filled with dechlorinated tap water and fed *ad libitum* with shrimp food (JBL Novo Prawn, Neuhofen, Germany). Fourth-instar larvae were used in all the experiments to maximise the chances of encountering robust cognitive abilities. In addition, larger larvae allow a more precise tracking. All animals were reared and manipulated according to ethics regulations applied in the European Union.

2.2. Experimental apparatus

The experimental apparatus consisted of two light sources, a camera, and a servo mechanism, which controlled the projection of the shadow of a square cardboard (shadow) above twelve 1.5 ml spectrophotometer plastic cuvettes (Z187992-1PAK, Sigma-Aldrich, Germany) where the larvae had been individually placed. One light source consisted of two LED panels (30 cm x 30 cm), located above the cuvettes (Fig. 1A). The second light source was an infrared backlight (880 nm) placed behind the cuvettes. In front of the cuvettes, a camera (acA 1300 – 60gc, Basler, Germany) equipped with a high-pass infrared filter (RG 850 Filter – 40.5 mm, Heliopan, US) recorded the experiments (for more details, see Dessart et al., 2023). The projected shadow induced naive larvae to dive vertically, escaping from potential danger. After repeated presentations of the shadow, the escape response decreased due to habituation, a form of non-associative learning (Dessart et al., 2023).

2.3. Experimental procedure

The experimental procedure included a training phase and a test phase. During the training phase, individuals were presented with a shadow 10 consecutive times (trials), separated by a specific inter-trial interval (ITI). The stimulus was a black cardboard square (16 cm side) attached to a wooden stick and fixed to a servomotor controlled by an Arduino Uno board. During a trial, the stick turned 100∘ and returned back to the resting position, during 3 s. After the 10th shadow presentation, the larvae rested for a specific period (retention time) before a final presentation of the shadow (test phase).

Two experiments were set up to evaluate the duration of memory and possible effects of ITI on the duration of memory. First, 6 treatments of 20——30 individuals were established, each trained with 2-min ITI and tested at different times after the training, ranging from 4 min to 24 h (i.e., Treatment 1 = 4 min, Treatment 2 = 30 min, Treatment 3 = 1 h, Treatment 4 = 2 h, Treatment 5 = 3 h, Treatment 6 = 24 h, Table 1). These intervals were chosen to investigate the memory persistence of mosquito larvae. The order of the treatments was pseudorandomised. Then, a new set of larvae were trained with 2 min ITI, 5 min ITI or 10 min ITI, and retention tested at 24-hour (i.e., Treatment 6 = 2 min ITI, Treatment 7 = 5 min ITI, Treatment 8 = 10 min ITI, Table 1). The order of the treatments was also pseudorandomised, and six replicates of 10 individuals were trained per day, resulting in 6 consecutive days of training (Table 1).

Training and testing took place in the afternoon, from 12 h to 19 h. Individuals were removed from the database if they transformed into pupae during the training or retention period (n = 1), if they remained motionless throughout the entire training period (n = 1) or if the tracking failed to distinguish the individual (n = 2) (Table 1). A total of 205 individuals from 21 replicates were kept for the analysis.

2.4. Data analysis

Each replicate was video recorded, and the individual trajectory was extracted using a tracking algorithm previously used by Dessart et al. (2023) (Fig. 1B). We first applied a detection rate by comparing the number of frames successfully identified by the tracking algorithm with the theoretical maximum number of frames. All videos were analysed with a minimum detection rate of 82.5 % (Table 1). Two analyses were then performed on individual trajectory.

To assess learning and memory abilities, we considered the stimulus response corresponding to the behavioural response of individuals during the 3-seconds trial period of the shadow passage (Fig. 1C). Using these data, we first excluded individuals that were at the bottom of the cuvette at the start of a trial (below 9/10th of the cuvette length, 26.1 % of trials removed, Table 2). We then calculated the variable Vertical

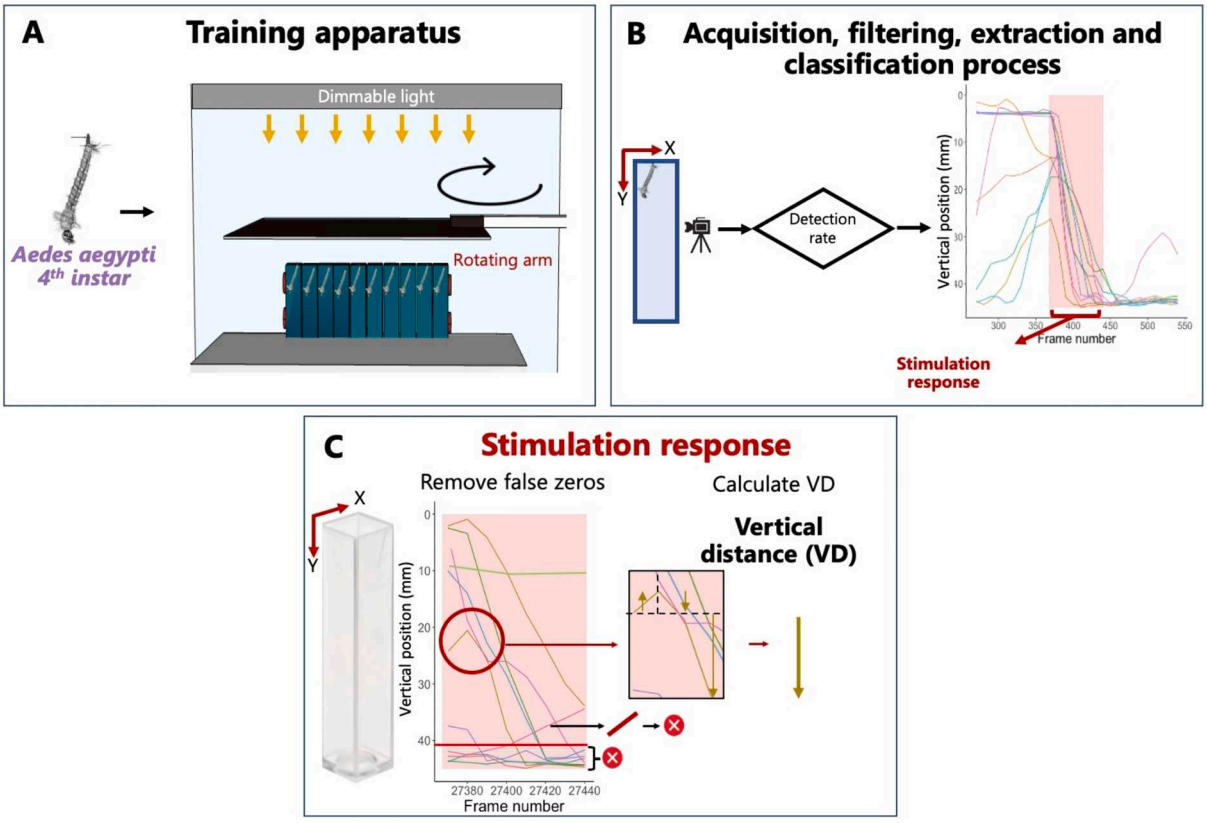

Fig. 1. Schematic of the experimental protocol. A) *Aedes aegypti* larvae were reared in the laboratory and trained on the apparatus at the fourth larval. B) Experiments were video-taped and individual trajectories were extracted. C) We analysed the behavioural response during the aversive stimulus, using the metric vertical distance (VD). This variable was quantitative and calculated as the relative sum of the distance travelled vertically towards the bottom of the cuvette. In addition, two filters were applied to exclude individuals that were at the bottom of the cuvette during the first few frames of the stimulus and individuals that moved upwards during the 3-second stimulus period.

Table 1
Details of the two experiments conducted. Each replicate represents 10 individuals (or less where indicated) trained during one session.

Replicate	Group	Retention	ITI	ID number	Detection rate	Vertical length (px)	Comment
1	1	4 min	2	10	0.82	399.4	
2	1	4 min	2	10	0.83	403.8	
3	1	4 min	2	10	0.95	401.6	
4	2	30 min	2	10	0.93	403.3	
5	2	30 min	2	10	0.92	400.5	
6	2	30 min	2	10	0.96	400.7	
7	3	1 h	2	10	0.97	400.6	
8	3	1 h	2	10	0.89	404.3	
9	3	1 h	2	9	0.93	401.1	ID#10 not tracked
10	4	2 h	2	9	0.98	406.6	ID#6 never moved at all
11	4	2 h	2	10	0.97	400.8	
12	4	2 h	2	10	0.92	405.6	
13	5	3 h	2	8	0.97	400.8	ID#7 transformed in pupae
14	5	3 h	2	10	0.9	399.7	
15	5	3 h	2	10	0.98	404	
16	6	24 h	2	10	0.98	401.9	
17	6	24 h	2	10	0.95	403.4	
18	7	24 h	5	9	0.85	403.6	ID#8 not tracked
19	7	24 h	5	10	0.99	400.8	
20	8	24 h	10	10	0.9	400	
21	8	24 h	10	10	0.92	401.8	

The retention column refers to the time between training and test. ITI = inter-trial interval used during the session. ID number corresponds to the number of individuals for each replicate. Detection rate was calculated as the ratio between the maximum number of frames and the actual number of frames identified by the tracking software. Vertical length was calculated as the difference between the maximum and the minimum individual position measured by the tracking software on each video.

Table 2
Summary of the filtering steps. Between 19 and 30 individuals were trained for each species.

2 MIN ITI						*5 MIN ITI*	*10 MIN ITI*	All
4 MIN	30 MIN	1H	2H	3H	24H	24H	24H	Total
30	30	29	29	28	20	19	20	205
320	330	319	319	308	220	199	220	2235
236	253	212	256	236	159	147	152	1651
26.3 %	23.3 %	33.5 %	19.7 %	23.4 %	27.7 %	26.1 %	30.9 %	**26.1 %**
230	250	205	255	235	158	140	149	1622
2.5 %	1.2 %	3.3 %	0.4 %	0.4 %	0.6 %	4.8 %	2.0 %	1.8 %
28.1 %	24.2 %	35.7 %	20.1 %	23.7 %	28.2 %	29.6 %	32.3 %	**27.4 %**

If the individual's position was near the bottom at the beginning of a trial, the response to that trial was removed, accounting for a total of 26.1% of removed trials. Individuals that moved upwards during a trial were also removed for that particular trial, accounting for 1.8% of trials.

Distance (VD) as the vertical downward distance travelled by each individual during the 3 s stimulus onset (Dessart et al., 2023). Using VD, we excluded individuals that travelled to the top of the cuvette during a trial (i.e., that travelled more than 10 mm upwards, 1.8 % of trials removed, Table 2). A total number of 205 individuals and 1622 trials were retained for the analysis (Table 2).

3. Statistical analyses

3.1. Data sharing

All the results were analysed using R version 4.1.1 (2021–08- 10) (https://cran.r-project.org/). The data and the R programs used in this study are available at: https://github.com/martindessart/Brain_Like_A_Sieve.

3.2. Data filtering

For all treatments, we verified that number of trials deleted by the criterion did not depend on the trial number (similar as Dessart et al., 2023). Briefly, we applied a Chi-square goodness of fit test to verify that the larval position did not change (i.e., increase or decrease) across trials.

3.3. Learning and memory performance

We first modelled learning performance using Generalised Additive Model (GAM) to provide a visual estimate of the training period. We defined models of increasing complexity and different smoothing functions and compared them using the GCV-UBRE in the *mgcv* package (Wood, 2017). We then evaluated the learning performance of each treatment by comparing the response in the 1st trial to the response in the 10th trial and in the Test phase. For each treatment, we used a linear mixed effects model, choosing VD as the response variable, trial as fixed factor and individual identity as random factor. We checked the homogeneity of the distribution of variances and residuals using the *DHARMa* package (Hartig, 2022). We evaluated the pairwise comparisons using the *emmeans* package with Tukey correction for 3 estimates (Lenth, 2021).

3.4. Test comparison

To compare the duration of memory across treatments, we compared the response in the Test phase by using a linear mixed effects model with VD as the response variable, the retention time as fixed factor and individual identity as random factor. We followed the same procedure as before to assess pairwise comparisons.

3.5. Learning efficiency

We also estimated how quickly larvae would significantly decrease their response as a function of the ITI, to estimate how many trials would be sufficient to induce a significant decrease in response. To answer, we compared the response at the 1st and the 2nd trial, using a linear mixed effects model as described above.

4. Results

4.1. Data filtering

To analyse the global position of the larvae over time, we compared the proportion of deleted trials by the two criteria between trials (similarly as Dessart et al., 2023). For each treatment, the deleted trials were not specific to a trial number: Treatment 1: $\chi^2 = 7.53$, df = 10, P = 0.674; Treatment 2: $\chi^2 = 3.6$, df = 10, P = 0.964; Treatment 3: $\chi^2 = 3.91$, df = 10, P = 0.951; Treatment 4: $\chi^2 = 6.81$, df = 10, P = 0.743; Treatment 5: $\chi^2 = 6.41$, df = 10, P = 0.780; Treatment 6: $\chi^2 = 9.68$, df = 10, P = 0.469; Treatment 7: $\chi^2 = 15.02$, df = 10, P = 0.131; Treatment 8: $\chi^2 = 6.93$, df = 10, P = 0.732.

4.2. Learning performance

For all treatments, the behavioural response of the larvae decreased with successive stimulations (Fig. 2, Fig. 4A). To model this behavioural response, the P-spline from GAM models was the best smoothing function, similar to as previous work (Dessart et al., 2023). For all treatments except Treatment 7, the Vertical distance VD was higher in the 1st than in the 10th trial: Treatment 1: t-ratio = 3.943, df = 45, P < 0.001; Treatment 2: t-ratio = 2.590, df = 46, P = 0.03; Treatment 3: t-ratio = 2.626, df = 37, P = 0.03; Treatment 4: t-ratio = 2.704, df = 49, P = 0.03; Treatment 5: t-ratio = 2.540, df = 46, P = 0.04; Treatment 6: t-ratio = 2.957, df = 30, P = 0.02; Treatment 8: t-ratio = 3.315, df = 28, P < 0.01 (Fig. 3A, Fig. 4B). For Treatment 7 (i.e., ITI = 5 min and retention time

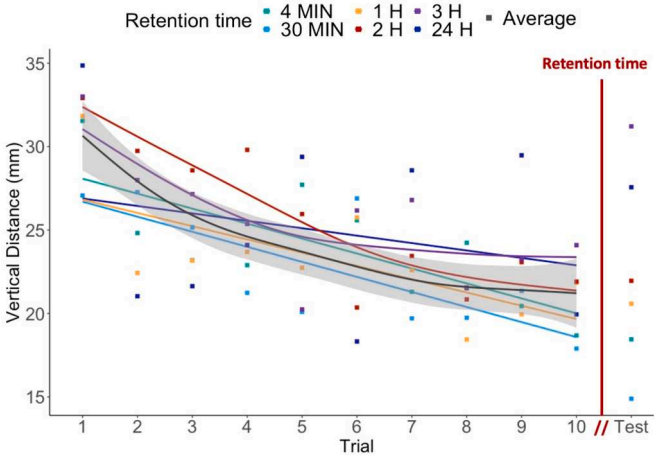

Fig. 2. Habituation curves for larvae trained with 2 min inter-trial interval and with several retention times. 4 min (green), 30 min (cyan), 1 h (yellow), 2 h (red), 3 h (purple), 24 h (dark blue) retention time. Average in black. Vertical distance (in millimetres) corresponds to the distance covered by an individual during the stimulus period, from the 1st to the test phase. Red vertical line corresponds to the retention time. Smoothing lines indicate the best-fitting GAM model. Grey shading indicates 95 % confidence interval for the average response. Points indicate mean values.

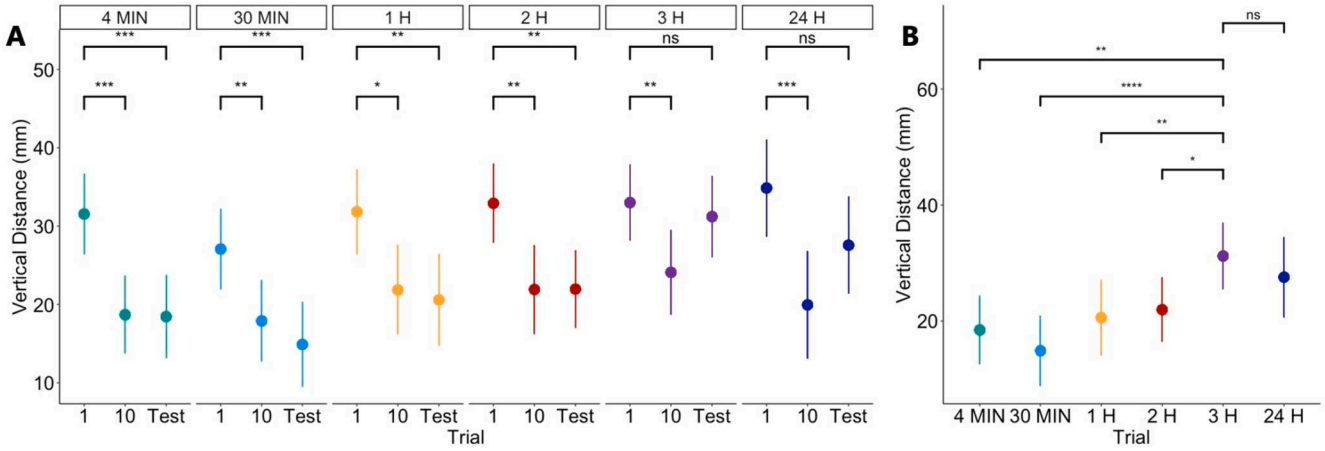

Fig. 3. Mosquito larvae retain visual information up to 2 h after habituation. A). For each retention time, vertical distance in millimetres travelled by individuals responding to an aversive stimulus during the 1st, the 10th and the test trial. B) Comparison of the vertical distance travelled by individuals during the test trial. 4 min (green), 30 min (cyan), 1 h (yellow), 2 h (red), 3 h (purple), 24 h (dark blue). Points indicate mean values and bars indicate +- 95 % confidence intervals. NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

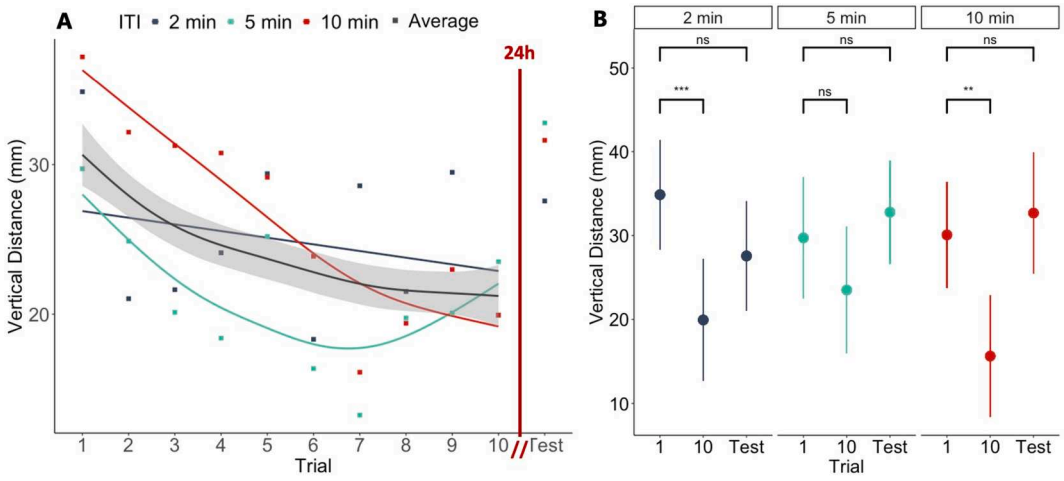

Fig. 4. Learning and memory performance of individuals is independent of the inter-trial interval. A) Habituation curves for individual training with 2 min (dark grey), 5 min (green), 10 min (dark red) inter-trial intervals. Average in black. Vertical distance (in millimetres) corresponds to the distance covered by an individual during the stimulus period, from the 1st to the test phase. Red vertical line corresponds to the retention time of 24 h. Smoothing lines indicate the best-fitting GAM model. Grey shading indicates 95 % confidence interval for the average response. B) For each inter-trial interval, the vertical distance in millimetres travelled by individuals responding to an aversive stimulus during the 1st, the 10th and the Test trial. Points indicate mean values and bars indicate +- 95 % confidence intervals. NS, not significant; **P < 0.01, ***P < 0.001.

= 24 h), although the response decreased after the 1st trial, the difference between the 1st and the 10th trial was not significant: t-ratio = 1.088, df = 32, P = 0.53 (Fig. 4B). This difference may be due to the small number of trials retained for this comparison (1st trial: n = 13; 10th trial: n = 12).

4.3. Effect of the retention time on memory performance

To investigate how long the information from the training would be stored in the mosquito larval brain, we compared the response during the Test phase for different retention times. First, we compared the response at the 1st trial to the Test phase (Fig. 3A). For retention times up to 2 h, the response in the Test phase was lower than in the 1st trial: Treatment 1: t-ratio = 3.823, df = 46, P < 0.01; Treatment 2: t-ratio = 3.354, df = 46, P < 0.01; Treatment 3: t-ratio = 2.529, df = 37, P = 0.04; Treatment 4: t-ratio = 2.906, df = 45, P = 0.02; Treatment 5: t-ratio = 0.520, df = 46, P = 0.86; Treatment 6: t-ratio = 1.533, df = 29, P = 0.30 (Fig. 3A). We also compared the response at the Test phase between the highest response (Treatment 5 = 3 h) with other Treatments (Fig. 3B). The response at the Treatment 5 was higher than Treatment 1: t-ratio = 3.097, df = 100, P < 0.01, Treatment 2: t-ratio = 3.874, df = 101, P < 0.001, Treatment 3: t-ratio = 2.366, df = 103, P = 0.02 and Treatment 4: t-ratio = 2.319, df = 98, P = 0.02. The response at the Treatment 6 was no different from Treatment 5: t-ratio = 0.704, df = 105, P = 0.48, Fig. 3B).

4.4. Effect of intertrial interval on long-term memory

As the larvae showed no retention after 3 h, we investigated whether 2 min ITI was sufficient to induce a long-term memory. We trained larvae using the same procedure, but with an increased ITI of 5 min and 10 min. We compared the response in the Test phase after 24 h of retention time for 3 ITI: 2 min, 5 min and 10 min. The difference between the 1st trial and the Test phase was not significant for any of the three Treatments: 2 min ITI: t-ratio = 1.533, df = 29, P = 0.29; 5 min ITI: t-ratio = -0.596, df = 28, P = 0.82; 10 min ITI: t-ratio = -0.550, df = 25, P = 0.85 (Fig. 4B).

4.5. Effect of intertrial interval on learning efficiency

To characterise the speed of learning, we compared the response between the 1st and the 2nd trials for the 3 ITIs. The difference between the 1st and the 2nd trials was only significant for the 2-min ITI: t-ratio = 2.955, df = 15, P < 0.01; 5 min ITI: t-ratio = 0.836, df = 13, P = 0.42; 10 min ITI: t-ratio = -0.069, df = 16, P = 0.94 (Fig. S1).

5. Discussion

In this work, we took advantage of an automated system to investigate memory persistence in the 4th instar of *A. aegypti* mosquito larvae. Two distinct questions were addressed: how long mosquito larvae could retain information following a habituation learning paradigm, and whether habituation training with longer inter-trial intervals would induce long-term memory in mosquito larvae or not. In the first part of this study, we found that following a habituation protocol, mosquito larvae could retain the information for up to 2 h after 10 trials with 2 min ITI. Yet, no memory was found after 3 h and 24 h of retention. In the second part of this work, we focused on the effect of ITI on the memory persistence at 24 h after training. Equally, we found no long-term memory at 2 min ITI, 5 min ITI and 10 min ITI.

These results support the hypothesis that the ecological context of mosquito larvae would favour short-term memory (Juliano, 2009). First, mosquito larvae live in a wide range of unpredictable environments, where visually hunting predators could be abundant, the structure of the habitat complex and water characteristics (e.g., temperature, light intensity, turbidity) are constantly changing (Crespo, 2011; Yee, 2016; Chandrasegaran et al., 2018). Similar to the crab *Pachygrapsus marmoratus*, which maintains a high response level to a potential flying predator, mosquito larvae could remain safe by quickly forgetting a previous habituation to a moving object, if this reveals to be innocuous (Tomsic et al., 1993). Similarly to *Neohelice granulata* crabs, previous work by our team has shown that habituation learning in *A. aegypti* larvae is context specific (Pientrantuono et al., 2021). However, here the larvae are not tied to a specific location in their aquatic environment and should not be able to learn differentially to less relevant stimuli over a long period of time.

Second, previous studies have shown that mosquito larvae can perceive a wide range of stimuli, such as light contrast, (Liu et al., 2022), conspecific alarm cues (Ferrari et al., 2008), predator vibrations (Roberts et al., 2019), and predator chemical cues (Chandrasegaran et al., 2018). As part of the neuston, these perceptual abilities, combined with their high speed and their diving reflex, allowed them to escape danger (Mellanby, 1958).

Third, when confronted with a repeated stimuli in the same modality, mosquitoes quickly adapt their behaviour in response to experience, i.e., they habituate in few trials. (Baglan et al., 2017; Dessart et al., 2023). Consequently, we can speculate that mosquito larvae should forget quickly and respond strongly to new stimuli after a few times. They should not invest in a costly long-term memory but should favour their own development to minimise time spent in water. This speculation seems interesting when considering the extensive studies on the cognitive abilities of adult mosquitoes, which can retain information for up to 72 h after visual conditioning (Chilaka et al., 2012) and for which remembering information about the last blood-meal is a crucial adaptative behaviour (Vinauger et al., 2014). Furthermore, while adults *A. aegypti* have been shown to learn to associate an a chemical cue with a blood-reinforced thermal stimulus (Vinauger et al., 2014), the question of the potential ability of mosquito larvae to perform associative learning remains open.

To distinguish habituation to other declines in behaviour, previous authors proposed ten criterions (Rankin et al., 2009). The first criterion define habituation. The second, the spontaneous recovery of the response if the stimulus is withheld, and the eighth, dishabituation, were verified in our previous study (Dessart et al., 2023). By comparing the response after training to several controls, we could rule out sensory adaptation, fatigue, and contextual effects. Here, by increasing the inter-trial interval to 5 min and 10 min, we observed a decrease in individual spontaneous activity during the training. However, the high individual response at the test phase after 24 h allowed us to rule out all these effects. By increasing the ITI, we also observed that individuals trained with a shorter ITI learned faster than individuals trained with a longer ITI, as revealed by a more rapid decrease in response magnitude along trials at 2 min as compared to others (Fig. S1).

This result supports the fourth criterion proposed by Rankin et al. (2009) for describing the behavioural characteristics of habituation, namely that an increase in stimulus frequency leads to an increase in response decrement.

Some other criteria remain to be explored in the habituation of mosquito larvae, for instance the third criterion which states that after repeated sessions of training, the decrement in response becomes more rapid or more pronounced (Rankin et al., 2009). The use of this criterion may explain how larvae can habituate more quickly to new stimuli shortly after a stimulus has previously appeared, and opens up new questions about the habitat and the cognitive abilities of mosquito larvae.

Finally, the standardised strain reared in the laboratory could influence the ability of *A. aegypti* ability to retain information. Previous authors have shown that a standardized strain could affect the learning abilities and individual fitness (Papaj et al., 1987; Nieberding et al., 2018). Our system is suitable for studying field-reared mosquito larvae and even other small aquatic species that show a comparable escape response.

In this work, we have seen that mosquito larvae have remarkable short-term memory after habituation to an aversive stimulus. This system is well suited to address fundamental questions about larvae abilities to learn and remember, to explore the underlying neurobiological mechanisms, and to ecological perspectives, such as the impact of pollutants or the role of the ecological structure in the development of cognitive abilities.

Funding

Financial support for this work was granted by Region Centre-Val de Loire, France APR IR 2020 COMPORTATE. COMPORTATE is supported by Réseau MiDi – Milieux et Diversité, Pole DREAM – French Water Team, Zone Atelier de la Loire – LTER France Loire, Réseau ENTOMO-CENTRE, Département d'Indre-et-Loire –Direction de l'Attractivité des Territoires (France).

CRediT authorship contribution statement

Martin Dessart: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Claudio R. Lazzari:** Conceptualization, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. **Fernando J. Guerrieri:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have shared the link to my data in the material and methods (line 246-247)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2024.104650.

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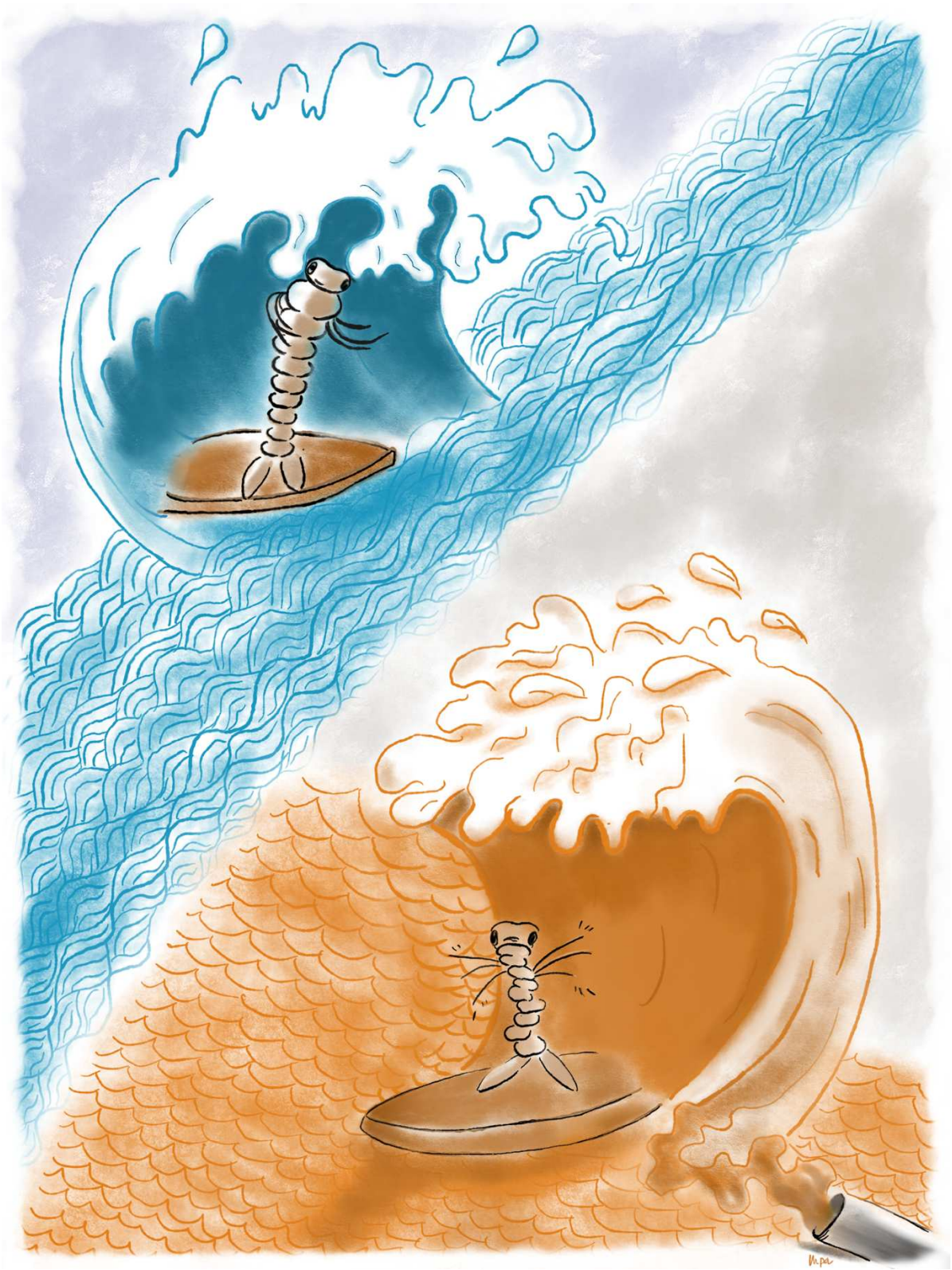

Martin DESSART

LA COGNITION DES LARVES DE MOUSTIQUE COMME INDICATEUR BIOLOGIQUE DE LA QUALITE DES ECOSYSTEMES AQUATIQUES

Résumé :

Dans les écosystèmes d'eau douce, les capacités des organismes sont altérées par les activités humaines. En particulier, l'effet de polluants à doses sous létales altèrent la perception, l'intégration et le stockage d'informations chez les invertébrés aquatiques. Pour l'instant, il est difficile de quantifier ces effets via des méthodes traditionnelles d'analyse chimique ou d'estimation de l'abondance et de la richesse spécifique. Alors, de nouveaux types d'indicateurs biologiques sont développés, avec la particularité d'utiliser les capacités cognitives comme des paramètres sensibles et physiologiquement intégratifs. L'objectif principal de ce travail de thèse est d'évaluer les capacités cognitives des larves de moustique en rapport avec leur environnement et d'examiner si elles peuvent être utilisées en tant qu'indicateur biologique de la qualité des écosystèmes d'eau douce. L'ensemble de nos résultats montrent que les larves de moustique sont capables d'apprendre et de mémoriser des informations et que ces capacités sont modulées par les conditions environnementales et la présence de polluants. Grâce à un cadre expérimental contrôlé, ce travail de thèse fournit une preuve de concept de l'utilisation de la cognition des larves de moustique comme indicateur de la qualité des écosystèmes d'eau douce.

Mots clés : apprentissage, mémoire, activité, écotoxicologie, insectes, écologie sensorielle

Summary:

Human activities affect organisms living in freshwater ecosystems. Sublethal doses of pollutants alter the perception, integration and storage of information in aquatic invertebrates. However, it remains difficult to quantify these effects using chemical analysis of water or biodiversity monitoring. Instead, the development of novel biological indicators involves the analysis of cognitive abilities as a sensitive and integrative endpoint. In this work, we assess the cognitive abilities of mosquito larvae in relation to their environment and investigate whether they can be used as biological indicators of the quality of freshwater ecosystems. Taken together, our results show that mosquito larvae are capable of learning and remembering information that are modulated by environmental conditions and the presence of pollutants. Thanks to a controlled experimental framework, this work provides a first proof of concept for the use of the cognition of mosquito larvae as a bioindicator of freshwater ecosystem quality.

Keywords: learning, memory, activity, ecotoxicology, insects, sensorial ecology