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Original Article

Insecticides Cypermethrin and Chlorpyrifos Exposure: Effects on Sperm Motility and Fertility in *Cyprinus carpio* (Cypriniformes, Cyprinidae)

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Abstract

The agricultural production of the Argentine Pampa is based on intensive cultures, and the most widely used insecticides correspond to cypermethrin (pyrethroid) and chlorpyrifos (organophosphate), among others. Sperm motility and fertility assessments are essential topics for biology and population dynamics studies on fish species. In this study the alterations of these endpoints after exposure to commercial formulation of both insecticides were used to analyze the effects on common carp sperm. After the *in vitro* exposure at nominal concentrations of 0.25, 0.5, 1, and 2 µg/L of cypermethrin, a significant reduction in motility in 0.5 µg/L concentrations was observed respect control group. The same nominal concentrations were tested for chlorpyrifos, and the same significative effect was detected at 2.0 µg/L. Fertilization rate was significantly reduced at concentrations from 0.5 µg/L cypermethrin exposure, and from 1.0 µg/L in the case of chlorpyrifos. Eggs LC_{50} / 24 h (95% confidence limit) exposed to cypermethrin was 0.57 µg/L (0.36 - 0.88), and exposed to chlorpyrifos was 2.45 µg/L (1.13-5.29). However, is still debatable how these insecticides impact on fish reproduction, using this *in vitro* scenario we found a clear reduction in sperm motility and fertility.

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INTRODUCTION

In the last decades, the traditional agricultural production of the Argentine Pampa region was quickly replaced by intensive cultures. The area used for cereal and oil crops production in the country increased from 24,941 ha in 2003 to 35,988 ha in 2021, an increase of 44% in this period (FAOSTAT 2022a), and it continues to increase nowadays. The increase in agricultural production was also reflected in an increase in the use of agrochemicals. Although insecticide consumption in Argentina stood at 4598 t in 2020, an increase of 31% compared to 30 years ago, in some years like 2010 consumption exceeded 10000 t, and the historical average between 1990-2020 is 6800 t (FAOSTAT 2022b). The most commonly used insecticides in soybean production correspond to the pyrethroid cypermethrin, the organophosphate chlorpyrifos (Butinof *et al*. 2014) and, by more than two decades, the already banned organochlorine endosulfan. Historically, concerns about the possible toxic effects of cypermethrin and chlorpyrifos have been investigated in the Argentine pampa (Jergentz *et al*. 2005; Marino and Ronco 2005; Carriquiriborde *et al*. 2007; Fernandez San Juan *et al*. 2023), in the Basin of the Paraná-Paraguay River (Etchegoyen *et al.* 2017), in the semi-arid region of Argentina (Mas *et al*. 2020) and even in the Patagonian region (Sánchez *et a*l. 2019).

Ambient concentrations

Most mammalian studies highlight the effects on sperm concentration, motility, and morphology induced by insecticide exposure (Desai *et al.* 2016; Sánchez *et al.* 2018). In fish, sperm motility is normally produced by the activation of the sodium-potassium ATPase channels (Na-K ATPase) (Martínez *et al*. 2010). The action of cypermethrin is known for the inhibitory effect on this channel at the nervous system level, however, is still unknown its effect on sperm. In different species of vertebrates, including fish, after pyrethroid exposure, sodium-potassium ATPase channels malfunction and remain depolarized (Hénault-Ethier 2015). The existing possibility that insecticide residues lead to sperm motility problems can directly result in a drop in the fertilization and hatching rates of fish. This can directly lead to ecological/biodiversity problems for the affected species.

Reproductive potential is a biological parameter that plays a critical role in the fisheries stock enhancement evaluations (Brown Peterson *et al*. 2011; Kowalski and Cejko 2019; Schulz *et al*. 2023). Fishery management, supported by the information obtained from the fertility assessment, makes it possible to understand the fish stock and population viability (Murua *et al*. 2003). On the other hand, the description of the reproductive strategies and the fertility evaluation are fundamental topics for the study of the biology and population dynamics of fish (Kant *et al*. 2016).

The common carp [*Cyprinus carpio (Linnaeus, 1758)*], used in aquaculture programs, requires artificial reproductive management to achieve synchronization of the maturity of the eggs and sperm and thus achieve high percentages of high-quality oocytes and fecundity rates (Nahiduzzaman *et al.* 2014; Park *et al.* 2017). However, mature carps capture from the wild is simple, and does not require hormonal induction to produce egg and sperm maturity. In the present study, we evaluated the effects produced by the *in vitro* exposure to commercial formulations of cypermethrin or chlorpyrifos insecticides, analyzing the effects on sperm motility and fertility of the common carp *C. carpio*.

MATERIALS AND METHODS

Fish were captured from Chascomús lagoon (35°35′29″S 58°01′28″O), Buenos Aires Province (Berasain and Argemi 2008). During November (spring season), mature males and females were registered, and sperm and oocytes samples were extracted.

Sperm collection

For the capture and maintenance of living organisms was used fyke net (Colautti 1998; Solari *et al.* 2016). Once the captures were made, fish were placed in trays, the water excess was gently removed from the body surface with absorbent paper and the sperm was extracted by soft stripping. The released sperm was collected with a dry plastic pipette to avoid contact with water, placing the samples in microtubes. For each male, a total number of 10 samples were collected, thus ensuring a minimum volume of 200 µL each. Samples were kept on ice (4ºC), and sperm sample was diluted (1:100) in an immobilization solution using a TBT buffer (94mM NaCl, 27mM KCl, 50mM glycine, 15mM Tris-HCl, pH 7.5) (modified from Volckaert *et al.* 1994).

Stock solution and toxicity of insecticides

To prepare and test the toxicity of investigated insecticides stock solutions of commercial formulations of cypermethrin and chlorpyrifos (Sherpa® and LorsBan 48E®, respectively) were prepared with the addition of 0.3% NaCl to induce activation of the sperm (Aydin *et al.* 2005; Nahiduzzaman *et al.* 2014). Subsequently, dilutions were made with the 0.3% NaCl solution to obtain the final nominal concentration tested for motility and fertility evaluation (0.25, 0.5, 1, and 2 µg/L). The nominal concentrations used in the present work were determined using as an approximate average concentration the maximum limits allowed by national legislation, where a concentration of 0.6 µg/L is established for cypermethrin and 0.7 µg/L for chlorpyrifos.

Motility analysis

A 2 µL sample of diluted sperm in TBT buffer was placed on a glass coverslip, which was activated by adding 5 µL of distilled water and 0.3% NaCl (control group), as well as for the four concentrations (0.25, 0.5, 1, and 2 µg/L) of commercial formulations of cypermethrin and chlorpyrifos. Motility analysis was performed with an Olympus bifocal microscope.
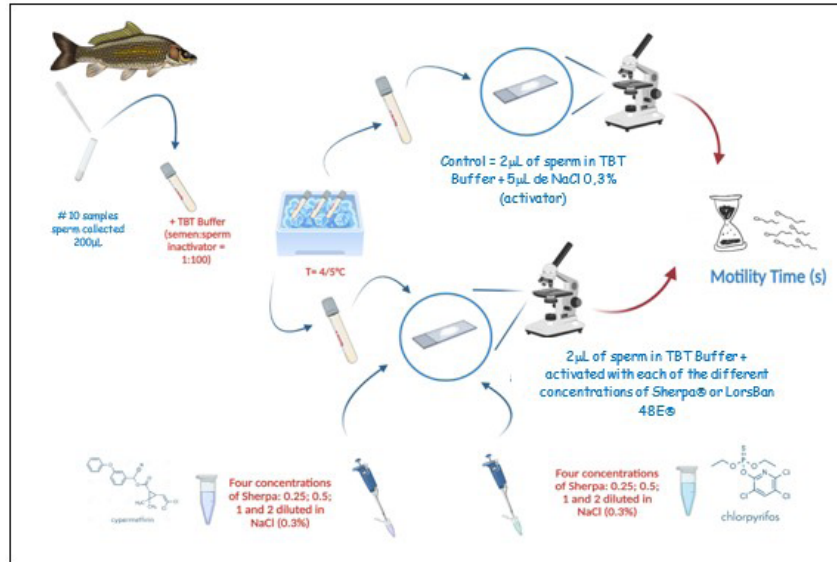
Figure 1. Summarizes the experimental design for the *in vitro* evaluation of cypermethrin and chlorpyrifos on the motility test time of *C. carpio* spermatozoa.

Sperm motility was quantified based on Betsy and Sampath (2014). The activation time was modified to establish less variability on the dataset. The average activity time was considered to establish less variability in the data. The total motility time was measured using a precision stopwatch (1/100) (Bozkurt *et al.* 2012) and adjusting for the average duration (Figure 2).
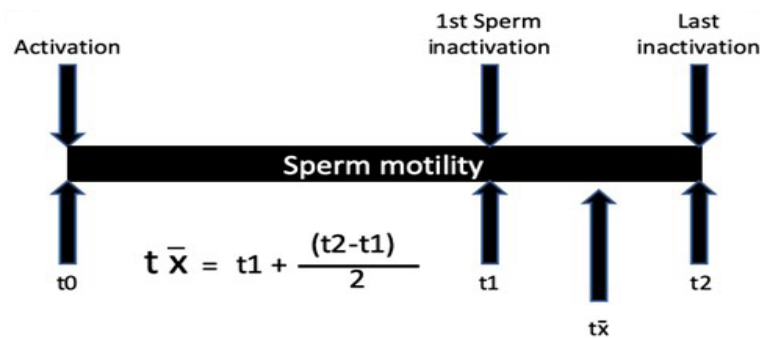
Figure 2. Model used to determine mean time in the sperm motility of *C. carpio.*
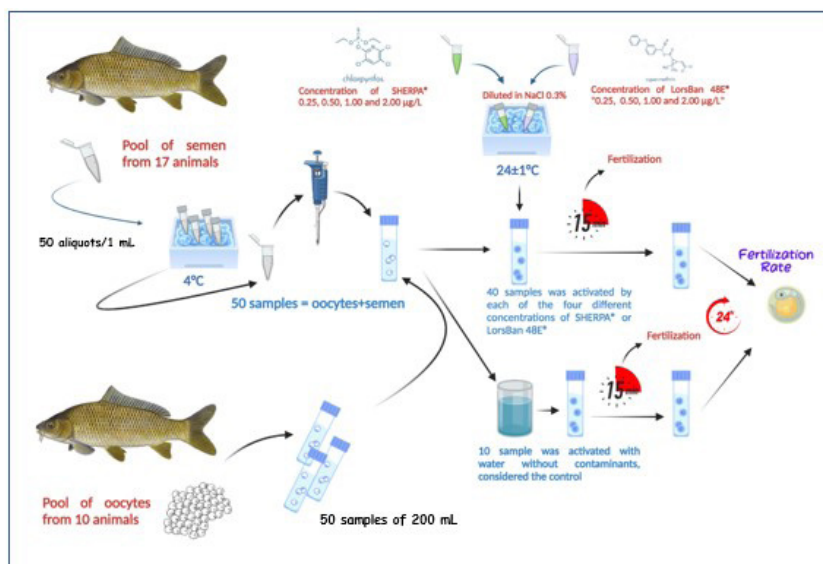
Fertilization rate analysis

An experimental design was followed to test whether pesticides produced deleterious effects on the fertilization rate, as shown in Figure 3.

During the above-mentioned semen collection, another pool of sperm samples from 17 common carps was collected and fractionated into 1 mL microtubes. Samples were kept on water/ice at 4°C in an immobilization solution (TBT buffer).

Ten females were collected and through slight abdominal pressure. The total volume of oocytes obtained was divided into 50 containers of approximately 200 mL each.

Following the artificial reproductive protocol for common carp by Woynarovich *et al.* (2011), each vial of sperm (1 mL) was mixed with the 200 mL of oocytes, and then the mixture was divided into 5 samples of 40 mL each. The activation solutions were added using a glass rod to homogenize the oocytes with semen (control), and commercial formulation with the insecticides. For oocyte fertilization, semen samples were used as described above.

Figure 3. Experimental design to evaluate the effect of cypermethrin or chlorpyrifos on the fertilization rate of *C. carpio*.

After the fertilization period of 15 min, washing was realized with the corresponding activation solution (with or without insecticides). This process was repeated three times, and the eggs were kept in the final activation solution for 1 hour (time of complete hydration in samples fertilized in control group). The egg adhesive secretions were removed using a tannin solution (0.5 g/L), which was mixed vigorously and washed 3 times (Woynarovich and Woynarovich 1980). Subsequently, 250 eggs were separated and placed in glass incubation bottles of 2 L. The culture system was established with 0.6 to 0.8 L / h recirculation, 24 ± 1 ºC, pH 7.68, and 12 D: 12N photoperiod.

After 24 h of incubation, the fertility rate (embryonic eggs) was evaluated by observing the eggs under a magnifying glass (AmScope 10X-40X) as described by Park *et al.* (2017).

Statistical analysis

Data obtained from motility time and fertility rate were submitted to analysis of normality (Shapiro Wilk and Kolmogorov-Smirnov) and of homogeneity of variance (Levene test). After meeting the statistical assumptions, the data were analyzed using a one-way analysis of variance (One-Way ANOVA), and the mean of treatments with pesticides was compared against the control group using Dunnet's test. Regression analyses were carried out to delineate the behavior of the response variables, concerning the pesticide concentrations with which the semen was placed in contact, with linear regression being the one that best fitted the observed behavior. Statistical analysis of the lethal effect was carried forward using PROBIT units (US EPA 1989) (Zar, 2010). Data were analyzed using GraphPad Prism 9.0 software.

RESULTS

Sperm motility decreased with the increased concentration of pesticides (Figure 4A), for the exposed group to cypermethrin, there was a reduction ($p<0.05$) in the motility time from the concentration of 0.50µg/L onwards compared to the control group. While in the control group, motility time was 126.6±13.50 seconds, there was a progressive reduction of 101.8±14.10 sec (0.50µg/L cypermethrin), 83.0±14.51 sec (1.00µg/L cypermethrin) and 48.80±13.99 sec (2.00µg/L cypermethrin). Chlorpyrifos exposure induced a reduction ($p<0.05$) in the motility time only in the sample exposed to the maximum concentration (2.00µg/L) 84.40±13.76 sec, being shorter than their control group with 124.4±16.09 sec.

The fertilization rate decreased as the concentration of pesticides increased (Figure 4B). Cypermethrin exposure resulted in significative reduction ($p<0.05$) of the fertilization rate at the lowest concentration utilized, compared with the control group. The fertility rate of the control group in this case was 69.86±5.58%, higher than that observed in other groups: 60.57±6.85% (0.25µg/L cypermethrin), 45.57±8.03% (0.50µg/L cypermethrin), 38.29±4.68% (1.00µg/L cypermethrin) and 36.57±7.61% (2.00µg/L cypermethrin). Chlorpyrifos produced a reduction ($p<0.05$) in the fertilization rate only in the highest concentrations. Samples exposed to 1.00 and 2.00µg/L of chlorpyrifos showed a fertilization rate of 62.14±6.44% and 47.71±8.24%, respectively, being lower than the control group (74.43±8.40%).

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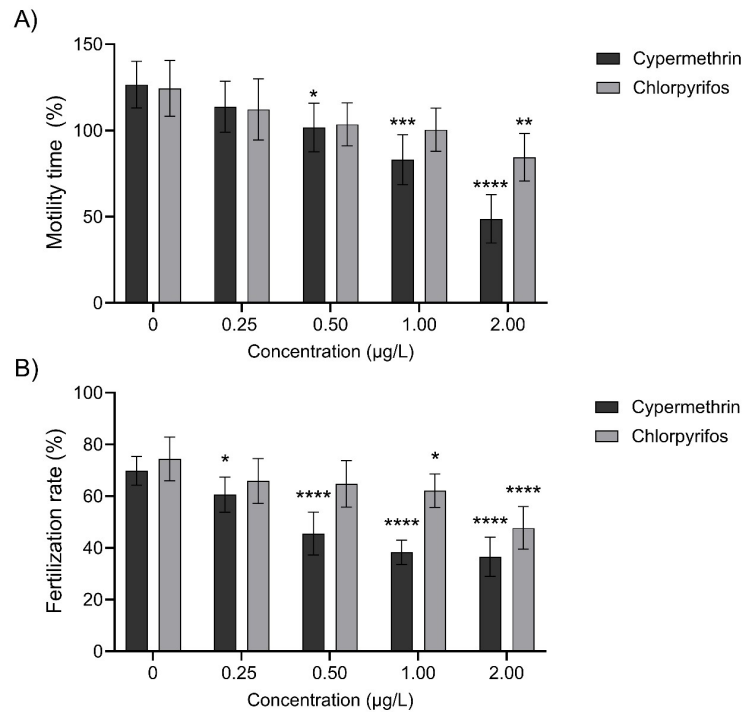
Figure 4. A) Sperm motility time (seconds) of common carp sperm activated with commercial cypermethrin and commercial chlorpyrifos. B) Fertilization rate (%) of common carp sperm activated with commercial cypermethrin or commercial chlorpyrifos. Data represent mean±SD of replicates. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 indicate significant differences with the control group (Dunnett test).

Figure 5 shows a negative linear correlation between motility time and fertilization rate with the concentrations of the two tested pesticides. As the insecticide concentrations increase, there is a decrease in motility time, and, consequently, the fertilization rate, showing a concentration-dependent toxicity of the pesticides.
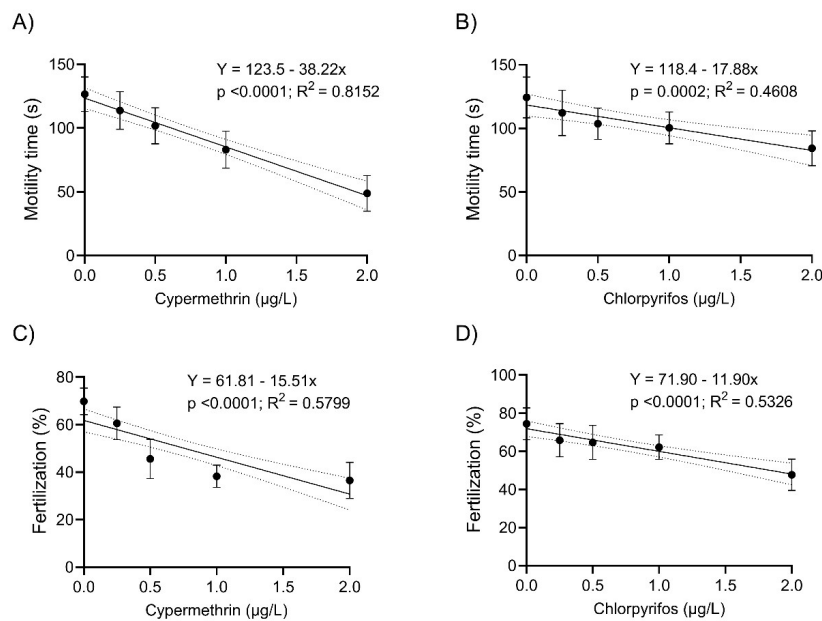
Figure 5. Regression equations of response variables concerning pesticide concentrations. A) Linear regression of motility time with cypermethrin concentration; B) Linear regression between motility time and chlorpyrifos concentration; C) Linear regression between fertilization rate and cypermethrin concentration; D) Linear regression between fertilization rate and chlorpyrifos concentration.

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DISCUSSION

In this study, we demonstrate sperm damage produced by cypermethrin or chlorpyriphos insecticides on fish reproduction, which are recurrently cited as the most affected vertebrate animal group. To our knowledge, this is the first report of alterations in fertilization of freshwater fish species, such as *C. carpio*, caused by these products. The results found with this species can be considered a model for other native and more sensitive species, where the impact of insecticide concentrations can be even more harmful to their reproduction.

We observed a decrease in the average motility time in the sperm samples that were activated with increasing concentrations of both insecticides. In the case of cypermethrin, the motility decreasing time occurred after exposure to a concentration of 0.50 μg/L of the insecticide. In the case of chlorpyrifos, the significant loss of motility time occurred only under the highest concentration tested (2 μg/L). According to Gallego and Asturiano (2019), sperm motility could be considered the main qualitative bioindicator of fish spermatozoa. In the present study, we did not assess the intensity of sperm motility after exposure to the two insecticides, as we standardized a minimum motility of 80% to perform the tests. On the other hand, the time of sperm motility is related to the opportunity for spermatozoa to fertilize an oocyte, the obtained results support understanding the effects of insecticides on the fertilization rate. Thus, when motility time decreases, a reduction in the fertilization rate is expected, which occurred when we exposed semen to concentrations above 0.25 μg/L of cypermethrin and 1.00 μg/L of chlorpyrifos.

The decrease in sperm motility time, when exposed to insecticides (cypermethrin and chlorpyrifos), is certainly related to the physiological mechanism of action imposed on the sperm cell by these products, even though they are different according to the molecular matrix which these insecticides originate. The physiological mechanism of cypermethrin as an insecticide is the inhibitory effect on the action of sodium channels in the nervous system (Hénault-Ethier 2015). The normal functioning of sodium channels occurs by allowing the passage of sodium when open, with the opposite happening when they are closed. This mechanism generates an action potential, which propagates down through the axon. When the nerve cell is exposed to pyrethroids, the sodium channels malfunction and may remain open, rather than returning to a closed state. This will lead to depolarization when exposed to type II pyrethroids (cypermethrin), causing tremors or involuntary movements and even muscle paralysis and death. The cellular physiology mechanism could explain the significant drop in the lifespan of *C. carpio* sperm exposed to concentrations above 0.50 μg/L. It seems quite evident that in these fish species with external fertilization -in which spermatozoa are immobile until the moment of release into the water-, the activation of the spermatozoa will be produced by a cell membrane potential, related to the osmolarity of the internal environment of the sperm (cell) and the external environment (water). In this case, a difference in the potential is generated for activating sodium-potassium ATPase channels (Na-K ATPase) located in the sperm tail region (Martínez *et al.* 2010; Elisio et al. 2015). In this sense, cypermethrin could alter the functioning of the sodium-potassium channels present in the sperm flagellum, compromising sperm motility and resulting in a decrease in motility time. In carp, K+ channel inhibitors markedly block sperm flagellar movement (Morisawa *et al.* 1983; Krasznai *et al.* 2000). Organophosphates are known as inhibitors of the enzyme Acetylcholinesterase. This enzyme acts in cells where acetylcholine (ACh) acts as a neurotransmitter and its inactivation leads to the accumulation of ACh, hyperstimulation of ACh receptors, and interruption of neurotransmission (Colovic *et al.* 2013), causing the death of insects. Some studies have already verified the presence of ACh in spermatozoa of some animals, such as fish, rabbits, bulls, and even in human sperm (Saiko 1969; Bishop *et al.* 1977; Alavi & Cosson 2006). Endosulfan is an organochloride insecticide extensively used in several countries to protect crops from pests. As several studies indicate, that endosulfan can affect human and animal development, Sánchez *et al.* (2018) demonstrated how sperm parameters and the process of chromatin decondensation could be altered by endosulfan in mice sperm. In the same work, a significant decrease in the percentage motility and viability of spermatozoa with respect to controls was found, and the ultrastructure analysis of sperm cells showed evident changes in the structure of the plasma and acrosome membranes of sperm incubated with endosulfan. Furthermore, it was verified that the inhibition of ACh receptors completely blocked sperm motility (Sliwa 1995), as well as the inhibition of the enzyme that synthesizes ACh intensely reduced this parameter in human semen. These studies confirmed the relationship between ACh and sperm motility of these species, and even though no studies are confirming the occurrence of this molecule in fish sperm, the reduction in motility time observed when they were exposed to the highest concentrations of chlorpyrifos leads to the belief that the insecticide is acting on some motility mechanism, possibly related to ACh.

The reduction in the fertilization rate of *C. carpio* was evident from 0.25 μg/L of cypermethrin. On the other hand, a concentration above 1.00 μg/L of chlorpyrifos resulted in significative loss in the fertilization rate, demonstrating a lesser negative effect of this product on fish spermatozoa. One explanation for this result is the acute toxicity of pyrethroids (such as cypermethrin) which is substantially higher for aquatic species than other pesticides (Lu *et al.* 2019). In our study, it was evident that a strong toxic process reduced the motility time and, consequently, the fertilization rate, being explicit in the experimental results with cypermethrin. According to Ögretmen *et al.* (2016), motility is the critical functional parameter in fish to determine the fertilization rate. More precisely, successful fertilization will depend on the movement of the spermatozoon in a straight line (Kholodnyy *et al.* 2020), which may be a circular movement, depending on the species. Sperm velocity is obtained by the distance between

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the initial and final points in the motility track due to the time spent on this movement. Thus, time ends up being decisive in the short period of sperm motility, resulting in the encounter with the oocyte and, consequently, the spermatozoon manages to penetrate it through the micropyle and fertilize it (Nagahama *et al.* 1983; Kudo 1991). Beirão *et al*. (2019), reinforce that the contact time between the oocyte and sperm is a determining factor for achieving high fertilization rates, and that it depends on two basic factors: sperm longevity (lifetime) and the time that the sperm oocyte will be receptive to be fertilized. The reduction in sperm motility time did not allow them to reach the oocytes and, consequently, fertilization could not have success.

According to the Subsecretaria de Recursos Hídricos de la Nación (2003, 2005) (Argentine environmental agencies), the acceptable limits of cypermethrin and chlorpyrifos in superficial water is 0.6 µg/L and 0.7 µg/L respectively. This concentration should be revised after this study, as the critical concentration of cypermethrin observed for the fertilization rate of *C. carpio* was 0.25 µg/L. In the soybean productive region of Argentina, the problem with cypermethrin seems to be getting worse due to the concentration of this pesticide detected in the water, causing concern for the ichthyofauna of the Paraná-Paraguay River basin, one of the most important in the region due to its biodiversity. In the study realized by Etchegoyen *et al.* (2017), the authors observed a triple of the concentration of cypermethrin in water in two years (from 0.24-0.74 µg/L). In the rivers of the Argentine pampa, although 100% of the samples from the study by Fernandez San Juan *et al.* (2023) contained cypermethrin, its average concentration was only 0.0056 µg/L, while for chlorpyrifos the average was 0.45 µg/L.

The positive relationship between both reproduction processes would explain a chain reaction, nevertheless, we cannot rule out that insecticides could produce adverse effects on oocyte viability, eggs, or even in embryos.

Both insecticides behaved similarly, producing a negative effect by reducing the reproductive capacity of both gametes, although a greater toxicity or greater impact of cypermethrin could be observed at lower concentrations.

LC_{50} determined to the species *Cyprinus carpio* was determined in 1,04 ng/L to animals of 4 cm approximately exposed to cypermethrin (Sarka and Saha, 2018) and 792 µg/L to the chlorpyrifos exposure with animals of 14 cm (Jaffer and Rabee, 2022). In this study we determined to the eggs LC_{50} / 24 h (95% confidence limit) exposed to cypermethrin was 0.57 µg/L (0.36 - 0.88), and exposed to chlorpyrifos was 2.45 µg/L (1.13-5.29), showing e highest sensibility of the eggs.
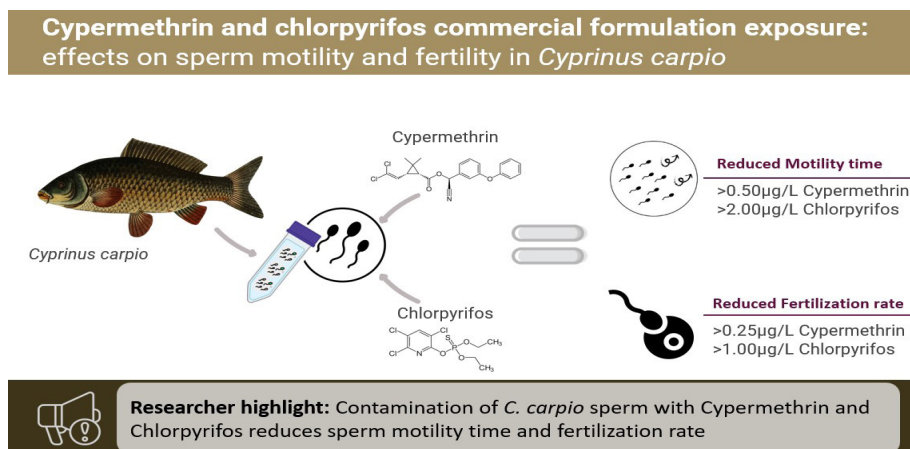
The increase in agricultural production Argentine Pampa region, basically in terms of soybean production, also reflected in an increase in the use of the agrochemical. The insecticides most used correspond to cypermethrin and chlorpyrifos. This situation requires an ecological risk analysis focused on their effect on the biota. According to Lu *et al.* (2019), toxicity benchmarks based on bioavailability must continue to be developed, especially since current bioavailability-based aquatic toxicity benchmarks are limited to a few combinations of pyrethroids in invertebrates, and most studies have only considered acute lethality as the endpoint limiting the application of bioavailability in risk assessment.

CONCLUSION

Exposure of *C. carpio* semen with insecticides such as chlorpyriphos and cypermethrin reduces sperm motility time and fertilization rate. Concentrations from 0.25 µg/L of cypermethrin and 1.0 µg/L of chlorpyrifos are critical for the fertilization of the oocytes of the species.

The *C. carpio* is well known opportunist and invasive species, very resistant to adverse conditions including high concentrations of contaminants like heavy metals or pesticides, as was demonstrated by other authors in the LC_{50}.

The national concentration limits established for the Subsecretaria de Recursos Hídricos de la Nación (2003, 2005) represent a very high-risk situation for the *C. carpio,* because it was demonstrated that it affects sperm motility and egg viability, putting in risk the population prosperity of this and other more sensitive species.

Cypermethrin and chlorpyrifos commercial formulation exposure: effects on sperm motility and fertility in *Cyprinus carpio*

Cypermethrin

Cyprinus carpio

Chlorpyrifos

Reduced Motility time
>0.50µg/L Cypermethrin
>2.00µg/L Chlorpyrifos

Reduced Fertilization rate
>0.25µg/L Cypermethrin
>1.00µg/L Chlorpyrifos

Researcher highlight: Contamination of *C. carpio* sperm with Cypermethrin and Chlorpyrifos reduces sperm motility time and fertilization rate

Figure 6. Commercial formulations of cypermethrin and chlorpyrifos reduce sperm motility time and fertilization rates in *Cyprinus carpio*.

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AUTOR CONTRIBUTION

FA, AP, FL conceived and planned the experiments. FA designed and performed the experiments. FA, DS, RBR, JLB contributed with the analysis of the results and to the writing of the manuscript.

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