CYPERMETHRIN INDUCED ALTERATIONS ON SEMINIFEROUS TUBULES OF *Dugesia gonocephala*, MACROINVERTEBRATE FROM THE CHILEAN ALTIPLANO

Omar Espinoza-Navarro, Camilo Arriaza, Carlos Cereceda and Héctor Rodríguez Bustos

SUMMARY

Cypermethrin is a synthetic pyrethroid widely used as an agropesticide. The aim of this study was to determine the effect of cypermethrin on reproductive patterns of the planarian Dugesia gonocephala*, a bioindicator species from Guacollo River, in the Chilean Altiplano, an ecosystem that is free of regular environmental pollutants. Specimens were subjected to doses of 1.455, 0.1455, 0.01455, 0.001455, 0.0001455 and 0.00001455mg·ml^{-1} cypermethrin (1/10, 1/100, 1/1000, 1/10000 and 1/1000000 LD_{50}, respectively). Planarians incubated in water from the same river were the control. The results showed that cypermethrin modified the morphology of the seminiferous tubules significantly increasing the area and perimeter of the tubules, also causing cellular changes, with a significant increase in (CD133$^{+)}$ stem cells. Is concluded that* D. gonocephala *is an excellent biomarker of environmental pollutants in aquatic ecosystems, and cypermethrin alters the architecture and cell proliferation of germ cells of the seminiferous tubules, possibly as a result of oxidative stress.*

Introduction

Pesticides are widely used in the control of agricultural pests. However, they are affecting significantly the quality of soils, water sources and humans who handle these products (Rodríguez and Sanabria, 2005). Cypermethrin is a synthetic pyrethroid derived from pyrethrin extracted from of chrysanthemum (*Chrysanthemum cinerarifolium*) bulbs and has a high insecticidal activity (Cantalamessa, 1993; Figueroa *et al*., 2011; Yuanxiang *et al*.; 2011).

The neurotoxic effect of cypermethrin resides in modifying voltage-dependent sodium channels in neuronal cells, causing a continuous depolarization (Anadon *et al*., 2009). In parallel, cypermethrin induces oxidative stress, causing disruptions or mutations in DNA, altering not only the architecture of tissues, but also their regeneration capacity. In general, these effects are considered as genotoxic (Henao *et al*., 2005; Patel *et al*., 2006).

The high altitude environment is usually associated with a weather of extremely low temperatures, low relative humidity, high levels of hypoxia, and low atmospheric pressure. These extreme conditions have been crucial in the adaptation of the species that inhabit high altitude regions, including the human species (Jacobsen *et al*., 2003; Espinoza-Navarro *et al*., 2011). The Chilean Altiplano has been considered as an 'ecological island', for having both endemic species and a habitat free of common pollutants. Species adapted to high altitude have a large variety of homeostatic mechanisms, such as adaptation to aquatic habitats, reduction of physical activity and development of a dehydration-resistant skin, among others (Raggi, 2000; Rodríguez *et al*., 2011).

Dugesia gonocephala is a fresh water organism that belongs to the phylum of Platyhelminthes, organisms that are the most geographically widespread in aquatic ecosystems. They are sensitive to environmental contaminants and therefore represent a useful potential biomarker model for toxicological research, due mainly the fact that they have an exceptional regeneration capacity, so they can be used for the study of differentiation and regeneration of lost or damaged tissues (Newmark and Sánchez-Alvarado, 2002; Salo and Baguna, 2002; Horvat *et al*., 2005; Sánchez-Alvarado, 2006).

Using immunohistochemistry, an antibody against the membrane protein CD133 (Prominin-1 in human and rodents) and its epitope, has served as a marker to identify various stem cell populations (Gurley and Sánchez-Alvarado, 2008). CD133 protein was isolated from mouse neuroepithelial stem cells and designated initially as prominin-1 (Shmelkov *et al*., 2005). It has been also studied in human cells, where the

KEYWORDS / Chilean Altiplano / Cypermethrin / *Dugesia gonocephala* / High Altitude / Pollution /

Received: 01/24/2014. Modified: 08/15/2014. Accepted: 09/02/2014.

Omar Espinoza Navarro. Biologist and Professor of Sciences, Universidad de Chile (UChile). Master in Education, Universidad de Tarapacá (UTA), Chile. Doctor in Celullar and Genetic Biology, Universidad Autónoma of Madrid, España. Professor, Universidad de Tarapacá, Chile. Address: Department of Biology, Faculty of Science, Universidad de Tarapacá. General Velásquez Nº 1775. Arica, Chile. e-mail: oespinoz@uta.cl

Camilo Arriaza O. Veterinarian and M. Sc. in Biology of Reproduction and Development, UChile. Professor, UChile. e-mail: carriaza@med.uchile.cl

Carlos Cereceda. Veterinarian, Universidad Santo Tomás, Chile. e-mail: ccereceda@ust.cl

Héctor Rodríguez Bustos. Veterinarian and Diplome in Education, UChile. Doctor in Cell Biology and Genetics, UAM, Spain. Professor, Uchile. Address: Institute of Biological Sciences, School of Medicine, UChile. Independencia 1027. Santiago, Chile. P.O.Box 8389100. e-mail: hrodrigu@med.uchile.cl

CIPERMETRINA INDUCE ALTERACIONES EN LOS TÚBULOS SEMINÍFEROS DE *Dugesia gonocephala*, MACROINVERTEBRADO PROVENIENTE DEL ALTIPLANO CHILENO

Omar Espinoza-Navarro, Camilo Arriaza, Carlos Cereceda y Héctor Rodríguez Bustos

RESUMEN

Cipermetrina es un piretroide sintético utilizado como agropesticida. El objetivo de este estudio fue determinar el efecto de la cipermetrina en los patrones reproductivos de la planaria Dugesia gonocephala*, una especie bioindicadora del río Guacollo, en el altiplano chileno, ecosistema libre de contaminantes ambientales. Los individuos fueron sometidos a dosis de 1,455, 0,1455, 0,01455, 0,001455, 0,0001455 y 0,00001455mg·ml^{-1} de cipermetrina (1/10, 1/100, 1/1000, 1/10000 y 1/1000000 LD_{50}, respectivamente). Las planarias del grupo control fueron incubadas en agua obtenida del mismo río. Los resultados muestran que cipermetrina modifica la morfología de los túbulos seminíferos, aumentando significativamente el área y el perímetro de los mismos, y además se observa un aumento significativo en el recuento de células madre (CD133^{+}). Se concluye que* D. gonocephala *es un excelente biomarcador de contaminantes ambientales en los ecosistemas acuáticos. Cipermetrina es un agropesticida que altera la arquitectura celular y la proliferación de las células germinales de los túbulos seminíferos, posiblemente como resultado de estrés oxidativo.*

CIPERMETRINA INDUCE ALTERACIONES EN LOS TÚBULOS SEMINÍFEROS DE *Dugesia gonocephala*, MACROINVERTEBRADO PROVENIENTE DEL ALTIPLANO CHILENO

Omar Espinoza-Navarro, Camilo Arriaza, Carlos Cereceda e Héctor Rodríguez Bustos

RESUMO

Cipermetrina es un piretroide sintético utilizado como agropesticida. El objetivo de este estudio fue determinar el efecto de la cipermetrina en los patrones reproductivos de la planaria Dugesia gonocephala*, una especie bioindicadora del río Guacollo, en el altiplano chileno, ecosistema libre de contaminantes ambientales. Los individuos fueron sometidos a dosis de 1,455, 0,1455, 0,01455, 0,001455, 0,0001455 y 0,00001455mg·ml^{-1} de cipermetrina (1/10, 1/100, 1/1000, 1/10000 y 1/1000000 LD_{50}, respectivamente). Las planarias del grupo control fueron incubadas en agua obtenida del mismo río. Los resultados muestran que cipermetrina modifica la morfología de los túbulos seminíferos, aumentando significativamente el área y el perímetro de los mismos, y además se observa un aumento significativo en el recuento de células madre (CD133^{+}). Se concluye que* D. gonocephala *es un excelente biomarcador de contaminantes ambientales en los ecosistemas acuáticos. Cipermetrina es un agropesticida que altera la arquitectura celular y la proliferación de las células germinales de los túbulos seminíferos, posiblemente como resultado de estrés oxidativo.*

CD133 antigen was also known as AC133 and identified as a marker of hematopoietic stem cells (Fargeas *et al*., 2004).

The seminiferous tubules are a very active tissue in cell mitosis and apoptosis because of the spermatopoietic process. It is for this reason that this tissue can be used for the study of acute cell damage produced by environmental contaminants such as cypermethrin.

The objectives of this study was to identify and measure the effect of cypermethrin, at similar concentration observed in areas of high exposure to these agropesticidas, on the morphometry and cellular activity in the seminiferous tubules of the testis of *Dugesia gonocephala*, an aquatic species from the Chilean Altiplano.

Material and Methods

Planarians

The collection of the freshwater planarian *Dugesia gonocephala* (Dugès, 1830), took place in the Guacollo River at the town of Visviri, Parinacota, Chile (17°35' 42.99"S and 69º28' 56.86"; 4100masl) during January 2010. The captured specimens were taken to the Laboratory of Reproductive Biology, Universdad de Tarapacá, Arica, where they were acclimated for 24h, keeping them in the same water of the Guacollo River as substrate, filtered through double gauze, and kept in an aquarium with forced oxygen at 18-24°C. Most zoologists classify the species *Dugesia* as a 'superspecies'. Kenk (1974), in his index of planarians species, determines a synonymy according to various localities of collection, establishing a natural habitat for *D. gonocephala* at 1800 to 4000masl. In the laboratory, specimens were fed with chicken liver (Oviedo *et al*., 2003; Richardson, *et al*., 2004).

The Ethic/Bioethics Committee of the Universidad de Tarapacá, approved the research protocols according to the principles of the 3Rs, to work with experimental animals.

Cypermethrin

The initial stock solution of cypermethrin (92.5% w/w; ANASAC, Chile) was prepared according to the intraperitoneal LD_{50} for mouse (485mg·kg^{-1} body weight). The equivalent LD_{50} for the planarian *D. gonocephala* was established as 14.55mg·ml^{-1}.

Experimental design

Seventy adult planarians >1.5cm long were selected and placed in sterile wide-mouth flasks of 50ml. They were distributed into seven groups (n=10) with six different dilutions of cypermethrin: 1/10, 1/100, 1/1000, 1/10000, 1/100000 and 1/1000000 of the LD_{50} (corresponding to concentrations of: 1.455, 0.1455, 0.01455, 0.001455, 0.0001455

and 0.00001455mg·ml^{-1}, respectively). The dilution was performed with water from the Guacollo River and a control group was kept in river water. Incubation in a volume of 10ml of each solution was at 18-24°C for 48h, considering that the renal bioelimination of cypermethrin in mammals is from 12 to 24h (Iannacone and Tejada, 2007; Crawford *et al*., 1981). Then the specimens were fixed in formalin for histological techniques and paraffin embedding. Sections of 5µm were mounted on previously xylanized glass slides.

Morphometric analysis

Mayer's hematoxylin-eosin stain was used. Analyses were performed under an Olympus CX31 microscope with integrated digital camera and 100× objectives and previous calibration made using a millimeter ruler. Micrographs were examined with the program Micrometrics SE Premium 2.0. Twenty seminiferous tubules of each individual were analyzed to determine the area and perimeter, based on a standard measurement for a longitudinal section of the animal, with evidence of the gut in the second third of the planarian.

Immunohistochemistry

The presence of CD133 (prominin in human and rodents) positive cells was analyzed with Antibody-Stem Cell Marker (ab19898, USA, as positive control human colon and in calibration dilution; negative control without antibody-stem cell marker). Twenty-five seminiferous tubules were observed and analyzed in each sample, with the same instrumental and program used for the morphometric analysis (HRP/DAB).

Statistical analysis

The area, tubular perimeter and number of stem cells positive for CD133 monoclonal antibody were tabulated in Microsoft Excel 2010 and the mean and standard deviation were calculated. The differences between the control group and those exposed to cypermethrin were analyzed statistically with Nonparametric ANOVA (Kruskal-Wallis test) and post test (Dunn's multiple comparison test) using GraphPad InStat 3 for Windows. Statistical significance was considered at $p<0.05$.

Results

At the time of histologic processing and microscope observation all animals had developed seminiferous tubules and were in sexually mature state conditions. Figure 1 shows a significant increase in the area of seminiferous tubules of *Dugesia gonocephala* in all groups incubated with cypermethrin with respect to the control in river water ($p<0.05$). The experimental group in the highest dilution (1/1000000) shows the largest increase in the tubular area compared to the control group (80000 *vs* 20000µm^2, respectively).

Figure 2 shows significant increases in the perimeter of the seminiferous tubules analyzed in all treated groups, with a greater circumference than that of the control group. The group kept at the lowest cypermethrin concentration (1/1000000 dilution) showed values of 1200µm, while 600µm were recorded for the control group.

Figure 3 shows the quantification of CD133 positive stem cells (neoblasts) in the periphery of the seminiferous tubules of *D. gonocephala* incubated in different dilutions of cypermethrin. All treatment groups showed significant increases in CD133 positive stem cells. It is emphasized that the group of lowest dilution has a higher average than the control group kept in river water (18 and 2, respectively).

Figure 4 shows a seminiferous tubule from the cypermethrin-treated group (1/1000000 dilution), with presence of CD133-positive stem cells inside the tubule. The epithelium

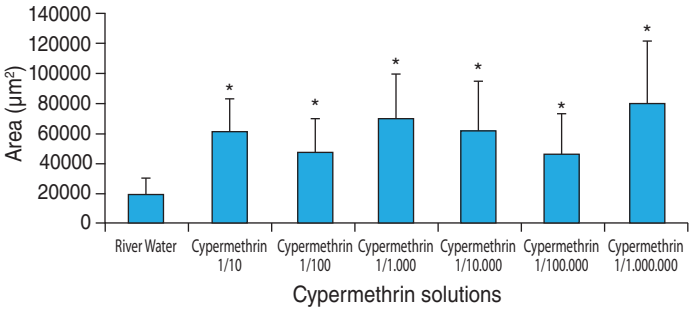

Figure 1. Changes in area of the seminiferous tubules of *D. gonocephala*, by effect of cypermethrin at doses of 1/10, 1/100, 1/1000, 1/10000, 1/100000, 1/1000000 of the LD_{50} (*: $p<0.05$).

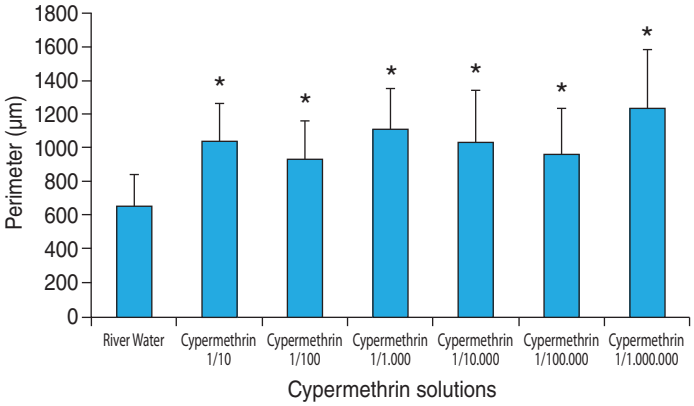

Figure 2. Changes in perimeter of the seminiferous tubules of *D. gonocephala*, by effect of cypermethrin at doses of 1/10, 1/100, 1/1000, 1/10000, 1/100000, 1/1000000 of the LD_{50} (*: $p<0.05$).

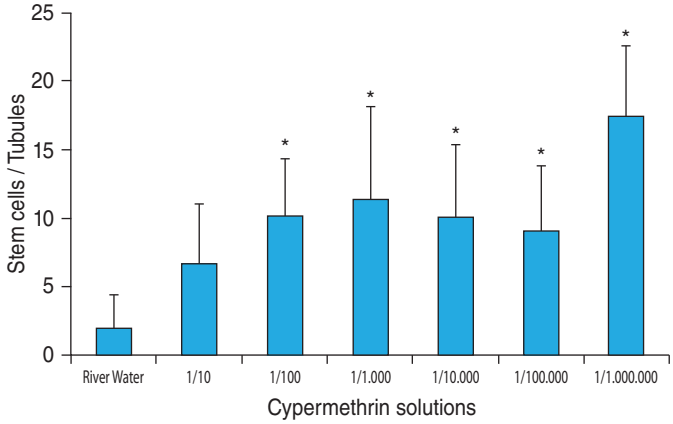

Figure 3. Cuantification of CD133-positive stem cells at doses of 1/10, 1/100, 1/1000, 1/10000, 1/100000, 1/1000000 of the LD_{50}. All treatment groups (except the group incubated at a dilution of 1/10) showed significant increases (*: $p<0.05$).

is disordered, with most gonial cells (or neoblasts) and scarce sperm cells in the tubule lumen.

Discussion

Most pesticides or environmental pollutants enter aquatic ecosystems, affecting the physiology and survival of species living in them. These genotoxic chemicals also generate teratogenic effects by their action on germ cells. An appropriate assessment of these damages requires suitable bioindicator agents. The

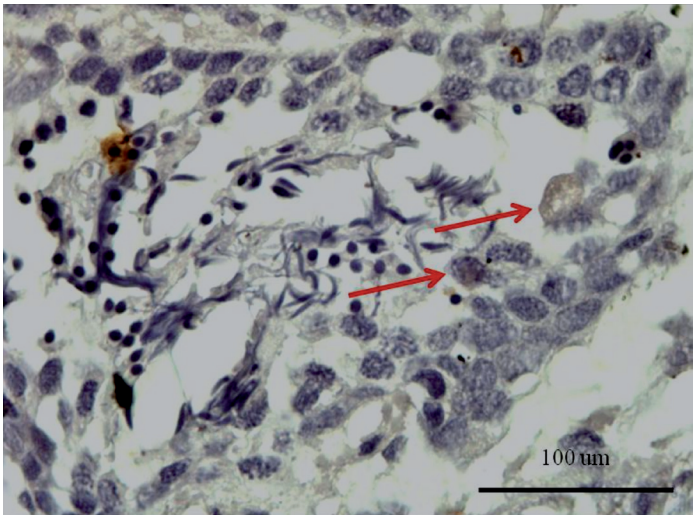

Figure 4. Seminiferous tubules treated groups (cypermethrin 1/1000000 dilution). Arrows show presence of CD133-positive stem cells inside the tubule (400x).

freshwater planarian *Dugesia gonocephala* is an important component of aquatic ecosystems in the Chilean Altiplano, it is sensitive to pollutants, has a high capacity of regeneration and is easily cultivated in the laboratory. In general, planarians are very useful experimental models (Chen *et al*., 1997; Salo and Baguna, 2002; Horvat *et al*., 2005).

The seminiferous tubules of the planarian *D. gonocephala* are composed of Sertoli cells, intertubular Leydig cells and germ cells that are arranged in a stratified manner (Menéndez-Valderrey, 2005; Alonso and Camargo, 2011).

In mouse, the seminiferous tubules incubated with the pyrethroid cypermethrin exhibit alterations in morphology and in cell proliferation causing changes on DNA and the mitotic process (Patel *et al*., 2006). In other studies in mice it has been shown that this pesticide induces severe histopathological alterations and interferes with spermatogenic activity. In *Drosophila melanogaster* it leads to extensive damage in the composition of DNA in a dose-dependent manner (Elbetieha *et al*. 2001; Mukhopadhyay, *et al*., 2004; Caneguim, *et al*., 2009; Salo *et al*., 2009; Wang *et al*., 2009; Al Hamdani and Yajurvedi, 2011). In general, these effects were attributed to a phenomenon of oxidative stress. Similar morphological changes were observed in seminiferous tubules of *D. gonocephala* treated with cypermethrin; an increase in the tubular area with an irregular, disorganized epithelium, and detachment of the tubular wall.

Planarians have the ability to regenerate any organ, as determined by the presence of totipotent stem cells in the stroma. Also, they have germ cells in seminiferous tubules and ovaries. Gashaw *et al.* (2007) have identified in human fetal seminiferous tubules the presence of $CD133^+$ cells during the second trimester of pregnancy.

The expression of $CD133^+$ stem cells in *D. gonocephala* was consistently higher in all groups treated with cypermethrin, the effects being dose dependents. Control animals show presence of $CD133^+$ cells in the periphery of the seminiferous tubule that probably correspond to neoblasts; in treated group the presence of $CD133^+$ cells is seen inside the seminiferous tubule, which might correspond to a proliferative response against oxidative stress caused by cypermethrin.

Neoblasts or undifferentiated cells may be multipotent stem cells and express the CD133 protein, the potential to differentiate into somatic or germ cells depending on whether microenvironmental conditions are favorable or not (Onal *et al*., 2012). Given the impact that pesticides have on the ecology and public health, the use of these products demand a better and sustainable management (Chirinos and Geraud-Pouey, 2011).

Conclusions

The results obtained indicate that the planarian *Dugesia gonocephala* from the Chilean Altiplano is an excellent biosensor (biomarker) to determine toxic effects on reproductive ecotoxicology.

The pesticide cypermethrin alters the cell structure of the seminiferous tubules, significantly increasing both the area and the tubular perimeter. It also increases the count of totipotent cells positive for the CD133 antigen. Overall, cypermethrin shows a dose dependent effect.

The high sensitivity of planaria to environmental conditions will allow a better assessment of the quality of waters where this species is found.

ACKNOWLEDGMENTS

The authors acknowledge the participation of Arnaldo Vilaxa Olcay, Universidad de Tarapacá, in capturing the specimens. This work was supported by the Major Research Project UTA Nº 4712-13 from the Universidad de Tarapacá.

REFERENCES

Alonso A, Camargo J (2011) The freshwater planarian *Polycelis feline* as a sensitive species to assess the long-term toxicity of ammonia. *Chemosphere 84*: 533-537.

Al-Hamdani N, Yajurvedi H (2011) Endocrine disruptive action of cypermethrin in male mice. *Toxicol. Environ. Health Sci. 3*: 69-79.

Anadon A, Martinez-Larrañaga M, Martinez M (2009) Use and abuse of pyrethrins and synthetic pyrethroids in veterinary medicine. *Vet. J. 182*: 7-20.

Caneguim B, Cerri P, Spolidório L, Miraglia S, Sasso-Cerri E (2009) Structural alterations in the seminiferous tubules of rats treated with immunosuppressor tacrolimus. *Reprod. Biol. Endocrinol. 7*: 19.

Cantalamessa F (1993) Acute toxicity of two pyrethroids, permethrin, and cypermethrin in neonatal and adult rats. *Arch. Toxicol. 67*: 510-513.

Crawford M, Crouche A, Hutson D (1981) Metabolism of cis-and trans-cypermethrin in rats. Balance and tissue retention study. *J. Agric. Food Chem. 29:* 130-135.

Chen A, Fink M, Letinski D, Barrett G, Pearsall J (1997) Residue of cypermethrin and its major acid metabolites in milk and tissue from dairy bovines treated with cypermethrin. *J. Agric. Food Chem. 45*: 4850-4855.

Chirinos D, Geraud-Pouey F (2011) El manejo de plagas agrícolas en Venezuela. Análisis y reflexiones sobre algunos casos. *Interciencia 36*: 192-199.

Elbetieha A, Da'as S, Khamas W, Darmani H (2001) Evaluation of the toxic potentials of cypermethrin pesticide on some reproductive and fertility parameters in the male rats. *Arch. Environ. Contam. Toxicol. 41*: 522-528.

Espinoza-Navarro O, Díaz J, Rodríguez H, Moreno A (2011) Effects of altitude on anthropometric and physiological patters in aymara and non aymara population between 18 and 65 years in the Province of Parinacota, Chile (3.700 masl). *Int. J. Morphol. 29*: 34-40.

Fargeas C, Joester A, Missol-Kolka E, Hellwig A, Huttner W, Corbeil D (2004) Identification of novel Prominin-1/CD133 splice variants with alternative C-termini and their expression in epididymis and testis. *J. Cell Sci. 117*: 4301-4311.

Figueroa R, Huerta de la Peña A, Pérez I, Marco V, López J (2011) Actividad insecticida de extractos de semilla de *Carica papaya* (L.) contra el gusano cogollero *Spodoptera frugiperda* (J.E. Smith) (*Lepidoptera: noctuidae*). *Interciencia 36*: 752-756.

Gashaw I, Dushaj O, Behr R, Biermann K, Brehm R, Rübben H, Rainer-Grobholz R, Schmid K, Bergmann M, Winterhager E (2007) Novel germ cell markers characterize testicular seminoma and fetal testis. *Mol. Human Reprod. 13*: 721-727.

Gurley K, Sánchez-Alvarado A (2008) Stem Cells in Animal Models of Regeneration. StemBook. The Stem Cell Research Community. Cambridge, MA, USA. http://www.stembook.org pp. 1-17.

Henao B, Palacio J, Camargo M (2005) Evaluación genotóxica de los plaguicidas cipermetrina y

diazinón en Tilapia roja (*Oreochromis* sp.). *Actual Biol. 27*(82): 43-55.

Horvat T, Kalafatic M, Kopjar N, Kovacevic G (2005) Toxicity testing of herbicide norflurazon on an aquatic bioindicator species - the planarian *Polycelis felina* (Daly). *Aqua. Toxicol. 73*: 342-352.

Iannacone J, Tejada M (2007) Empleo de la regeneración de la planaria de agua dulce *Girardia festae* (Borelli, 1898) (Triclacida: dugesiidae) para evaluar la toxicidad del carbofurano). *Neotrop. Helminthol. 1*: 7-13.

Jacobsen D, Rostgaard S, Vásconez J (2003) Are macroinvertebrate in high altitude streams affected by oxygen deficiency? *Freshw. Biol. 48*: 2025-2032.

Kenk R (1974) *Index of the Genera and Species of the Freshwater Triclads (Turbellaria) of the World.* Smithsonian Contribution to Zoology Nº 183. Smithsonian Institution Press, Washington, DC, USA. pp. 21-28.

Menéndez-Valderrey J (2005) *Las Planarias, los Gusanos Planos*. Asturnatura.com: Nº 50. www.asturnatura.com/articulos/planarias/inicio.php

Mukhopadhyay I, Chowdhuri D, Bajpayee M, Dhawan A (2004) Evaluation of in vivo genotoxicity of cypermethrin in *Drosophila melanogaster* using the alkaline comet assay. *Mutagenesis 19*: 85-90.

Newmark P, Sánchez-Alvarado A (2002) Not your father's planarian: A classic model enters the era of functional genomics. *Nature Rev. Genet. 3*: 210-220.

Oviedo N, Newmark P, Sánchez-Alvarado A (2003) Allometric scaling and proportion regulation in the freswater planarian *Schmidtea mediterranea*. *Devel. Dynam. 226*: 326-333.

Onal P, Gru D, Adamidi C, Rybak A, Solana J, Mastrobuoni G, Wang Y, Rahn H, Chen W, Kempa S, Ziebold U, Rajewsky N (2012) Gene expression of pluripotency determinants is conserved between mammalian and planarian stem cells. *EMBO J. 31*: 2755-2769.

Patel S, Pandey A, Bajpayee M, Parmar D, Dhawan A (2006) Cypermethrin-induced DNA damage in organs and tissues of mouse: Evidence from the comet assay. *Mut. Res. 607*: 176-186.

Raggi L (2000) El Altiplano Chileno, Guía para el Turismo Aventura y Turismo Científico. Centro Internacional de Estudios Andinos. Universidad de Chile. pp. 20-21

Richardson G, Robson C, Lang S, Neal D, Maitland N, Collins A (2004) CD133, a novel marker for human prostatic epithelial stem cells. *J. Cell Sci. 117*: 3539-3545.

Rodríguez D, Sanabria M (2005) Efecto del extracto de tres plantas silvestres sobre las Rizoctoniosis: la mancha sureña del maíz y los patógenos que la causan. *Interciencia 30*: 739-744.

Rodríguez H, Espinoza-Navarro O, Silva I, Needham D, Castro M, Sarabia L, Inostroza J, Jimenez L (2011) The effect of paraoxon on spermatogenesis in *Dugesia gonocephala* from the Chilean Altiplano: proliferation and apoptosis. *Environ. Sci. Pol. Res. 18*: 497-502.

Salo E, Baguna J (2002) Regeneration in planarian and other worms: new findings, new tools, and new perspectives. *J. Exp. Zool. 292*: 528-539.

Salo E, Abril J, Adell T, Cebriá F, Eckelt K, Fernández-Taboada E, Handberg-Thorsager M, Iglesias M, Molina M, Rodríguez-Esteban G (2009) Planarian regeneration: achievements and future directions after 20 years of research. *Int. J. Develop. Biol. 53*: 1317-1327.

Sánchez-Alvarado A (2006) Planarian regeneration: Its end is its beginning. *Cell 124*: 241-245.

Shmelkov S, Clair R, Lyden D, Rafii S (2005) Molecules in focus: AC133/CD133/Prominin-1. *Int. J. Biochem. Cell. Biol. 37*: 715-719.

Wang X, Liu S, Sun Y, Wu J, Zhou Y, Zhang J (2009) Beta-cypermethrin impairs reproductive function in male mice by inducing oxidative stress. *Theriogenology 72:* 599-561.

Yuanxiang J, Shanshan Z, Yue P, Linjun S, Liwei S, Weiping L, Zhengwei F (2011) Cypermethrin has the potential to induce hepatic oxidative stress, DNA damage and apoptosis in adult zebrafish (*Danio rerio*). *Chemosphere 82*: 398-404.