Dumancic, M.M., J.G. Oakeshott, R.J. Russell, and M.J. Healy 1997, Genetics 35: 251-270; Facon, B., B.J. Genton, J. Shykoff, P. Jarbe, A. Estoup, P. David 2006, Trends in Ecology and Evolution 21: 130-135; Galego, L.G.C., C.R. Ceron, and C.M.A. Carareto 2004, Revista UNORP: Saúde 3: 97-114; Galego, L.G.C., C.R. Ceron, and C.M.A. Carareto 2006, Genetica 126: 89-99; Galinkin, J., and R. Tidon 2000, Resumos do XXIII Congresso Brasileiro de Zoologia, 273-274; Gõni, B., P. Fresia, M. Calviño, M.J. Ferreiro, V.L.S. Valente, and L. Basso da Silva 2001, Dros. Inf. Serv. 84: 61-65; Gupta, J.P., 1970, Proceedings of the Indian Science Academy 36B: 62-70; Karan, D., S. Dubey, B. Moreteau, R. Parkash, and J.R. 2000, Genetica 108: 91-100; Linde, K., G.J. Steck, K. Hibbard, J.S. Birdsley, L.M. Alonso, and D. Houle 2006, Florida Entomologist 89(3): 402-403; Machado, T., A.M. Solé-Cava, J. David, and B.C. Bitner-Mathé 2005, Ann. Soc. Fr. 41: 7-13; Miller, M.P., 1997, Tool for population genetic analyses – TFPGA – 1.3: A Windows program for the analysis of allozyme and molecular population genetic data. Computer software distributed by author; Moraes, E.M., R. Mateus, and F.M. Sene 2000, Abstract book – XXI International Congress of Entomology, 271; Nascimento, A.P., and H.E.M. Campos Bicudo 2002, Genetica 114: 41-51; Nei, M., 1978, Genetics 89: 583-590; Parkash, R., and J.P. Yadav 1993a, Hereditas 119: 161-173; Parkash, R., and J.P. Yadav 1993b, Dros. Inf. Serv. 72: 150-152; Russell, R.J., G.C. Robin, P. Kostakos, R.D. Newcomb, T.M. Boyce, K.M. Medveczky, and J.G. Oakeshott 1996, Insect Biochem. Mol. Biol. 26: 235-247; Santos, J.F., T.T. Rieger, S.R.C. Campos, A.C.C. Nascimento, P.T. Felix, S.V.O. Silva, and F.M.R. Freitas 2003, Dros. Inf. Serv. 86: 92-95; Strauss, S.Y., J.A. Lau, and S.P. Carroll 2006, Ecol. Lett. 9: 357-374; Swofford, D.L., and G.J. Olsen 1990, Phylogeny reconstruction. In: *Molecular Systematics* (Hillis, D.M., and C. Moritz, eds.). Sinauer Associates, Inc., Sunderland, MA; Tidon, R., D.F. Leite, and B.F.D. Leão 2003, Biological Conservation 112: 299-305; Toni, D.C., P.R.P. Hofmann, and V.L.S. Valente 2001, Biotemas 14: 71-85; Tsacas, L. 1985, Annls. Soc. Ent. Fr. 21: 343-344; Vilela, C.R., 1999, Dros. Inf. Serv. 82: 37-39; Vilela, C.R., E.P. Teixeira, and C.P. Stein 2000, Mosca-africana-do-figo, Zaprionus indianus (Diptera: Drosophilidae). In: *Histórico e impacto das pragas introduzidas no Brasil. Editora Holos, São Paulo.* (Vilela, E., R.A. Zucchi, and F. Cantor, eds.), 48-52.

Toxicological tests of tibolone in *Drosophila melanogaster* wild type and Oregon-*flare* strains.

Castañeda-Partida, L.,[1] A. Silva-Galicia[1], O.F. Jiménez[1], C.F. Román[1], A. Silíceo[1], A. Durán-Díaz[2], I.E. Dueñas-García[1] and M.E. Heres-Pulido[1]. [1]Lab. Genética Toxicológica, [2]Matemáticas. Dept. Biología. FES Iztacala-UNAM. Av. de los Barrios 1. Los Reyes Iztacala, Tlalnepantla. Edo. de México, México. E-mail*: lauracp@campus.iztacala.unam.mx*

Introduction

For many years estrogen replacement has been used effectively to prevent osteoporosis and other climacteric complains such as vaginal dryness, hot flushes and mood symptoms that are related to the marked decline in plasma estradiol levels in postmenopausal women. Estrogen replacement, however, may lead to cell proliferation in the uterus and breast. In particular in the uterus, unopposed estrogen replacement treatment leads to an increased risk of developing uterine cancers. Therefore, an ideal therapy would be one that acts as an estrogen on the bone and the urogenital system, but not on the uterus or the breast (Bloom, 2006).

Tibolone (Livial, Org OD 14), produced by Organon (West Orange, NJ), is a synthetic steroid that has estrogenic, androgenic and progestagenic properties. It has been used in many countries for almost two decades, primarily for the prevention of postmenopausal osteoporosis and beneficial effects on menopausal and postmenopausal vasomotor, bone, vaginal and mood symptoms without affecting the endometrial, breast or cardiovascular systems (Falany *et al.,* 2004). Tibolone itself has no biological activity; its estrogenic, progestagenic and androgenic properties are the result of the activity of its metabolites on various tissues. After administration, tibolone is quickly metabolized into 3α-hydroxytibolone (3α-OH-tibolone) and 3β-OH-tibolone compounds, which are also present in an inactive, sulfated form (Modelska and Cummings, 2002). Sulfation is the major conjugation pathway involved in tibolone metabolism and may be significant in determining the tissue-specific effects of tibolone and its metabolites by modulating activity in situ. In general, sulfation inhibits the biological activity of steroidal compounds by prevention of binding to hormone receptors. Sulfation of tibolone and its metabolites is proposed to have an important role in regulating their tissue-specific effects. Selective inhibition of sulfatase activity by tibolone and its metabolites has been proposed as a mechanism for the specific effects of tibolone in breast and bone cells. Inhibition of sulfatase activity would decrease the conversion of the sulfates of tibolone and its metabolites to their unconjugated active forms. Also, the presence of specific sulfotransferase (SULT) isoforms in different human tissues may be involved in regulating tibolone activity in a tissue-specific manner (Falany *et al.,* 2004). A third compound, the Δ4-isomer, is formed from tibolone directly or from the 3β-OH-metabolites. The 3α- and 3β-OH-metabolites bind solely to the estrogen receptor (ER), whereas the Δ4-isomer has affinity for progesterone receptor (PR) and androgenic receptor (AR), but not ER (Modelska and Cummings, 2002). Since tibolone causes an increase in bone mineral density and is effective in the reduction of climacteric complaints but has no estrogenic effect on the endometrium, it is, therefore, considered to be the first member of a unique class of compounds described as selective, tissue estrogenic activity regulators or STEARs (Kloosterboer *et al.,* 2003). The concentrations of tibolone metabolites and the metabolic regulation of hormonal activities vary depending on tissue type. Tibolone given orally (2.5 mg) is rapidly absorbed, appearing in the plasma within 30 min and peaking in 4 h. Tibolone is metabolized mainly in the liver and is excreted in the urine and feces. The elimination half-life is approximately 45 h (De Gooyer *et al.,* 2001; Modelska and Cummings, 2002).

From 1996 to 2001 Beral *et al.* (2003) studied a million women between 50 and 64 years old confirming that 184 out of 18,186 ingesting tibolone developed breast cancer; besides that, the million women study revealed that this synthetic estrogen increased cancer risk in 1.45%. Other effects of tibolone in postmenopausal women, such as its influence on lipid metabolism, hemostasis, and sexual function, are less certain. In addition, the long-term effects of tibolone, particularly in reducing fractures, breast cancer, and cardiovascular disease, are still unknown (Modelska and Cummings, 2002).

The fruit fly *Drosophila melanogaster* is a multicellular eukaryote widely used in scientific research. It requires simple facilities, inexpensive culture media, it has a short generation time (about 10 days at 25ºC), it breeds a large number of individuals per generation, and *in vivo* assays can be done easily. *Drosophila* has also gained importance as a biological model in short term tests for toxicity screening of natural and synthetic compounds (Lewis *et al.,* 1998; Heres *et al.,* 2005; Dueñas *et al.,* 2005; Castañeda *et al.,* 2001). On the other hand, approximately 80% of human genes have a genetic homologue in *Drosophila melanogaster*. Most human genes are duplications and elaborations of their insect equivalents. In fact, not only individual domains and proteins but entire complexes and metabolic pathways are conserved between *Drosophila* and *Homo sapiens.* The knowledge from studying these *Drosophila* genes and the biological processes in which they

participate contributes to our understanding of the mechanisms of action of their human counterparts (Mackay; 2006; St. John and Xu, 1997).

With the concerns involving the use of estrogens in hormonal replacement therapy, there is a renewed interest in evaluating their potential side effects due to long-term use in postmenopausal women. In order to provide more information on tibolone toxic effects, we conducted toxicological tests in third instar larvae of *Drosophila melanogaster* wild type and Oregon-*flare* strains with regulated and highly constitutive CYP450 levels, respectively. This difference would indirectly indicate whether CYP450s are involved in tibolone metabolism in these *Drosophila* strains or not.

Materials and Methods

Chemical compounds

Tibolone (Livial, Organon), (Figure 1).

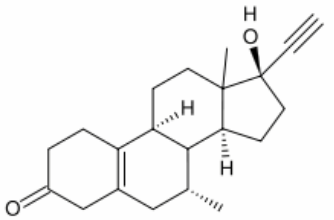

Figure 1. Molecular structure of tibolone.

Strains

Wild type (+/ +) *Drosophila melanogaster:* with regulated cytochrome P450 (CYP450) levels. Oregon-*flare* (ORR(1)/ ORR(2); *flr3/In(3LR)TM3, BdS)*: with highly constitutive CYP450 levels.

Toxicological tests

Adult flies were raised at 25ºC and aged in culture bottles containing mashed potato flakes medium and a conservative solution (Dueñas *et al*., 2001). Eggs from both strains were collected separately by shaking the flies into bottles (250 ml) containing an approximately 5 cm layer of fermenting fresh baker's yeast supplemented with sucrose (Graf and van Schaik, 1992). The egg collection bottles were then kept undisturbed in the dark for 8 h at 25ºC and a relative humidity of 65%. After removing the parental flies, the egg collection bottles were taken back to the incubator with the same conditions. Three days later, the 72 h ± 4 h, third instar larvae were washed out of the bottles with tap water at room temperature through a fine-meshed stainless steel strainer and thoroughly washed free of yeast while still in the strainer. Ten larvae were transferred to vials containing 0.5 g of mashed potato flakes prepared with 2 ml of Tibolone (Livial, Organon. CAS Nº 5630-53-5, 99% purity) at 0, 0.07, 0.156, 0.312, 0.625, 1.25 and 2.5 mg (therapeutic dose) in distillated water. Three replicates were made for each concentration in three independent chronic experiments for each strain. The treatment vials were kept at 25ºC and a relative humidity of 65% until pupation. The surviving flies were collected in alcohol 70% from the vials on days 10 to 12 after egg laying to quantify mortality. The mortality rates were plotted against tibolone concentration in order to calculate fitting by regression. Results were analyzed with one-way analysis of variance (ANOVA) where F test was calculated for statistically significant differences between concentrations and strains.

Results

Figures 2 and 3 show the mortality rates of Oregon-*flare* and wild type (+/ +) third instar larvae exposed to tibolone. There was not a dose-response effect in any of the strains and mortality rates were not higher than 15-20%.

The ANOVA results for the Oregon-*flare* strain showed there was no interaction between control and experimental treatments ($P = 0.2487$). In contrast, the ANOVA results for the wild (+/+) strain showed statistically significant differences between experimental treatments ($P = 0.037$); however, it is not possible to assure there is a biological explanation to this because of the standard

deviations. Finally, the ANOVA results between strains did not yield significant differences (P = 0.1315).

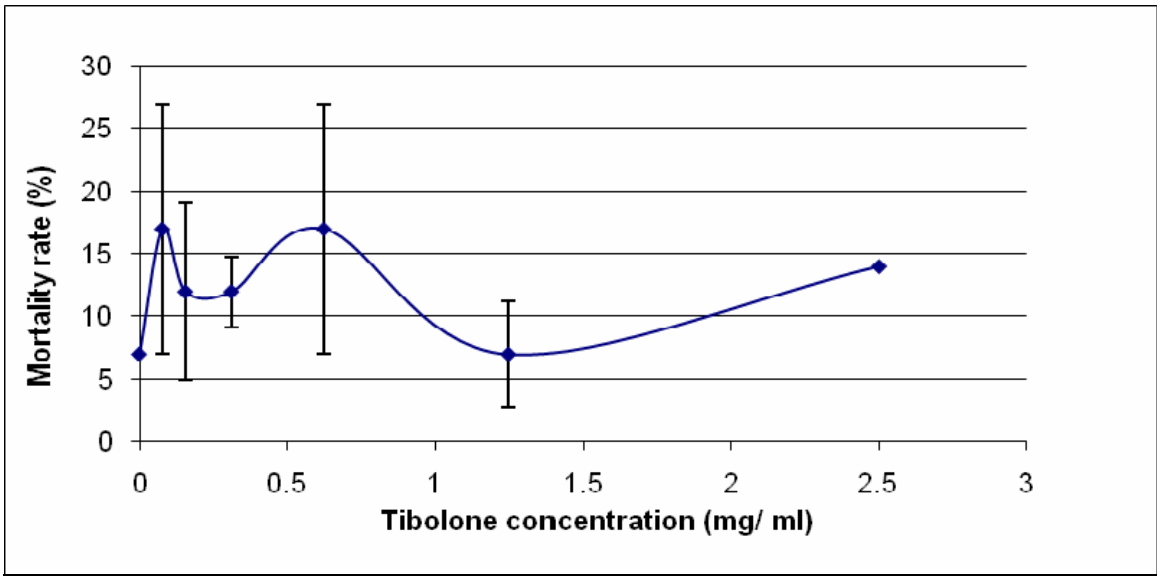

Figure 2. Mortality rate in Oregon-*flare* third instar larvae exposed to tibolone.

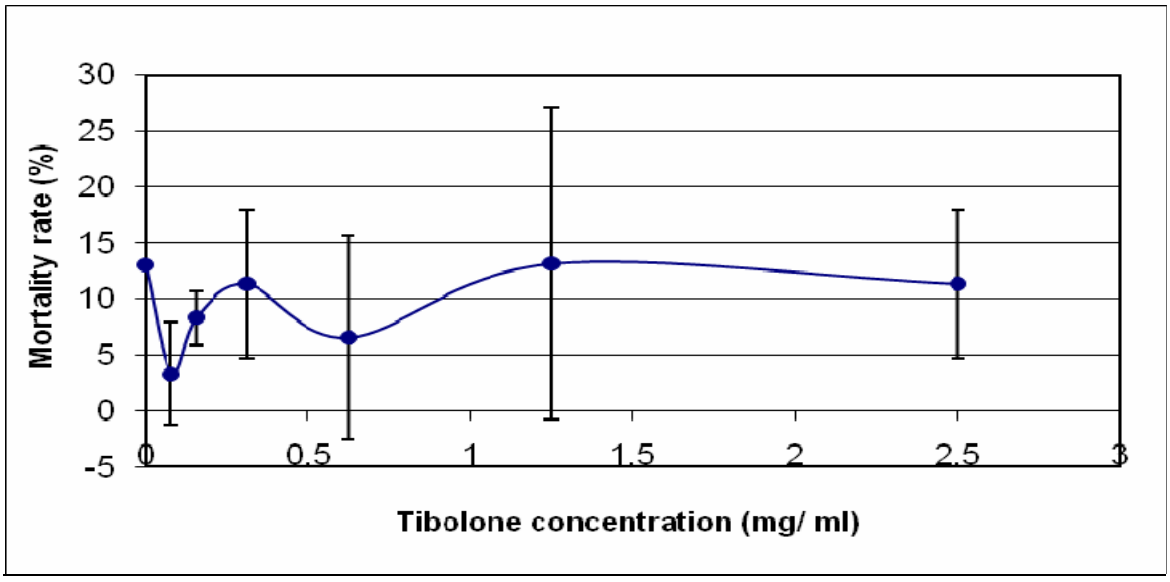

Figure 3. Mortality rate in wild type (+/+) third instar larvae exposed to tibolone.

Figure 4 shows the mortality curves for both *Drosophila* strains displaying a "mirror" image.

The main explanation to this would be the regulated CYP450 levels in the wild type (+/+) strain and the highly constitutive levels of these drug metabolizing enzymes in the Oregon-*flare* strain. All along the tested concentrations of tibolone the strains seem to respond in a different manner with respect to each other in three different moments and not showing a dose-response effect. We did not conduct experiments with tibolone concentrations higher than 2.5 mg, the therapeutic

dose, in order to keep our objectives in an actual toxicological perspective. Unfortunately, the ANOVA results and the standard deviations do not allow to assure this "mirror" image represents a biological process in answer to tibolone exposure in these different *Drosophila* strains.

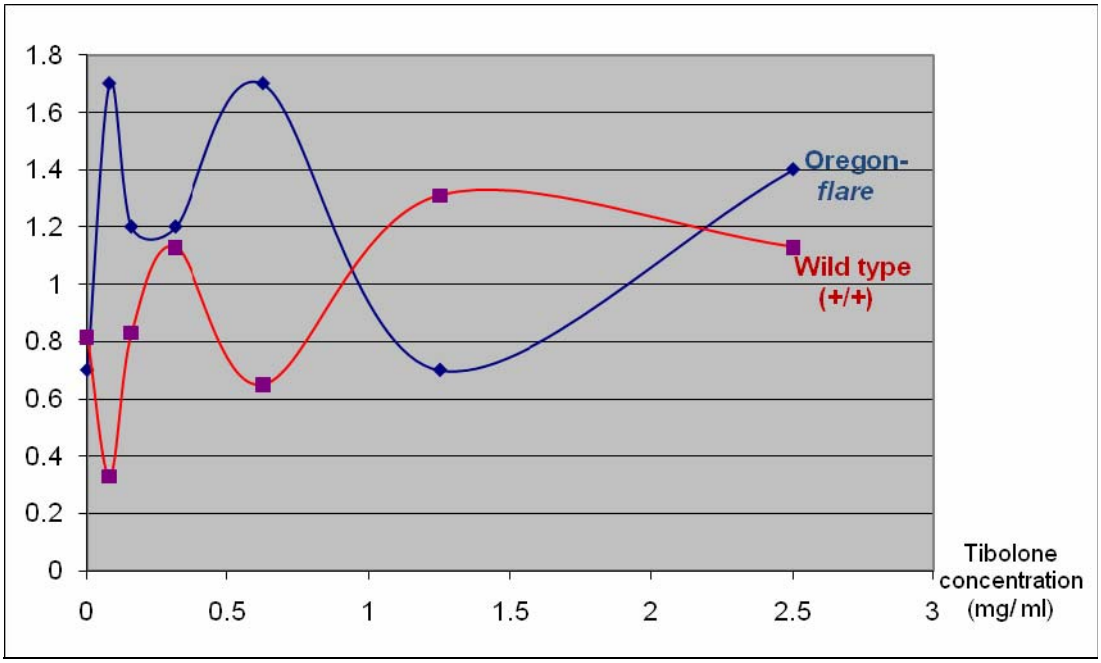

Figure 4. Mortality curves for Oregon-*flare* and wild type (+/+) third instar larvae exposed to tibolone.

Discussion

Toxicological tests were performed for toxicity screening of tibolone, a synthetic estrogen widely used in hormonal replacement therapy. Third instar larvae of the *Drosophila* Oregon-*flare* and wild type (+/+) strains were chronically exposed to the compound until pupation. The results seemed to show differences that could be accounted for by the CYP450 levels in these strains. From this point of view, the lowest concentration would have been biotransformed into a toxic metabolite by the constitutive CYP450s in Oregon-*flare* causing a relative mortality. In the case of the wild type (+/+) strain with regulated CYP450 levels, the lowest concentration of tibolone would have not been enough to trigger a cellular response (*i.e.*, CYP450s synthesis). Should this be true, the CYP450 enzymes would be involved in tibolone metabolism in *Drosophila* and not Phase II sulfotransferases (SULTs) that regulate tibolone in humans (Falany *et al.*, 2004). However, the standard deviations do not validate this idea, proving there would be another metabolic pathway in *Drosophila* common to both strains and without a dose-response effect which is very interesting.

There also exists the question whether tibolone was actually ingested by the larvae and absorbed as a xenobiotic or just as one more molecule in the diet. It is well known estreoids do not exist in invertebrates; in turn, they present ecdysteroids that perform hormonal functions: metamorphosis modulation (Terashima *et al*., 2005; Kozlova and Thummel, 2002), embryonic development and oogenesis (Terashima *et al.,* 2005). Ecdysteroids are produced by the prothoracic glands or ring glands located in the brain (Terashima *et al*., 2005; Kozlova and Thummel, 2002).

One of the main ecdysteroids is ecdysone, the metamorphosis hormone that regulates embryonic development.

Anyhow, if tibolone was ingested and/or absorbed by the larvae, we do not know its metabolism in *Drosophila*. Therefore, we can only suggest it was possibly processed in the fat body and the Malpighian tubules, the insect organs associated with metabolism, the ability to respond to xenobiotics and excretion (King-Jones *et al*., 2006; Yang *et al.,* 2007). Many of the response genes to xenobiotic compounds are highly expressed in these organs, leading to the opinion that they play similar roles to those of the liver and kidney in mammals (McGettigan *et al.,* 2005; Yang *et al*., 2007). It is precisely in these organs where gen *drh96* expresses, belonging to a superfamily of hormone nuclear receptors (Fisk and Thummel, 1995); the gen codifies receptor DRH96, a molecule exclusively found in the nucleus, related to transcriptional response to xenobiotics; mutant strains for this gene show certain resistance to phenobarbital (King- Jones *et al.,* 2006). Even more, many of the genes that regulate DRH96 codify diverse members of detoxifying enzymes that are conserved from insects to humans: cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), carboxylesterases, and UDP-glucuronosyl transferases (UGTs) (King-Jones *et al*., 2006).

In humans are present the *Steroid and Xenobiotic Receptor* (SXR*)* and the *Constitutive Androstane Receptor* (CAR) ortholog transcription factors to DHR96. These bind directly to endogen as well as to exogen lipophilic compounds to promote transcription of genes that codify Phase I and II enzymes, such as CYP450s, GSTs, UGTs, and SULTs (King- Jones *et al.,* 2006).

In view of these physiological similarities between organs and other molecular structures of insects and mammals, it is possible that tibolone had been absorbed and processed by similar metabolic pathways.

In women, tibolone activation is attributed to sulfation carried out by certain SULTs of Phase II (Falany *et al*., 2004). The parental molecule is sulfated (inactive form) mainly in the liver and distributed through the blood vessels to all the body (De Gooyer *et al.,* 2001); and since sulfation inhibits the biological activity of chemical compounds by preventing their binding to their receptors (Falany *et al.,* 2004), the titers of SULTs in the cells of a given tissue are decisive for the activation, explaining tibolone´s tissue specificity (De Gooyer *et al.,* 2001). It is thought tibolone has no activity on mammary tissue, because sulfatase activity is inhibited there (Falany *et al*., 2004; Modelska and Cummings, 2002).

So there is a minimal probability that tibolone has a toxic/genotoxic effect in mammary tissue cells which could lead to cancer. However, tibolone ingestion may extend at least a decade and, therefore, is more likely to cause some renal pathology.

Every day, each kidney filters approximately 1,700 L of blood, concentrating refuse residues in almost 1 L of urine with constant exposure to toxic substances that make them susceptible to lesions. Actually, nephropathy or nephritis can derive from excessive consumption of drugs such as analgesics (MedlinePlus, 2007).

Conclusion

Tibolone, at therapeutic doses, did not result toxic to *Drosophila* Oregon-*flare* and wild type third instar larvae, producing low mortality rates between 15-20% without a dose-response relation. This is good news for premenopausal and postmenopausal women undergoing chronical hormonal replacement therapy; however, special attention still must be paid to its effects on uterus, mammary glands, liver, and kidneys among other organs and systems.

Finally, it can not be affirmed from our data that the CYP450 enzymes are involved in tibolone metabolism, because no statistically significant differences were observed between

Drosophila Oregon-*flare* and wild type strains; the resulting standard deviations do not allow to clearly establish the biological effect of this synthetic estrogen on the larvae.

References: Beral, V., (Million Women Study Collaborators) 2003, The Lancet 362: 419-27; Blom, M.J., M. Groot Wassink, M.E. De Gooyer, A.G.H. Ederveen, H.J. Kloosterboer, J. Lange, J.G.D. Lambert, and H.J.Th. Goos 2006, J. of Ster. Bio. and Mol. Biol. 101: 42–49; Castañeda, P.L., G.L.E. Muñoz, D.A. Durán, P.M.E. Heres, and G.I.E y Dueñas 2001, Dros. Inf. Serv. 84: 44-47; De Gooyer, M.E., G.T. Overklift Vaupel Kleyn, K.C. Smits, A.G.H. Ederveen, H.A.M. Verheul, and H.J. Kloosterboer 2001, Mol. and Cel. Endocr. 183: 55-62; Dueñas, I.E.; M.E. Heres, P.L. Castañeda, and U. Graf 2002, Dros. Inf. Serv. 84: 166; Dueñas-García, I.E., V. Vega-Contreras, J.C. Gómez-Luna, L.F. Santos-Cruz, L. Castañeda-Partida, A. Durán-Díaz, and M.E. Heres-Pulido 2005, Dros. Inf. Serv. 88: 83-89; Falany, J.L., N. Macrina, and C.N. Falany 2004, J. of Ster. Bio. and Mol. Biol. 88: 383-391; Fisk, G.J., and C.S. Thummel 1995, Proc. Natl. Acad Sci., 92: 10604-10608; Heres-Pulido, M.E., G. Ávila-Acevedo, H. Dionicio-López, G.L.B. Hernández-Portilla, L. Castañeda-Partida, A.M. García-Bores, G. Martínez-Cortés, S.L. Cárdenas, G. Cerón, K. Tavera-Briseño, A. Vargas-Vera, and I.E. Dueñas-García 2005, Dros. Inf. Serv. 88: 103-107; Graf, U., and N. van Schaik 1992, Mutat. Res. 271: 59-67; King-Jones, K., M.A. Horner, G. Lam, and C.S. Thummel 2006, Cell Metabolism 4: 37-48; Kloosterboer, H.J., and A.G.H. Ederveen 2003, J. Steroid Biochem. Mol. Biol. 83: 157–165; Kozlova, T., and C.S. Thummel 2002, Development 129: 1739-1750; Lewis, D.F.V., C. Loannides, and D.V. Parke 1998, Environ. Health Pespect. 106: 633-641; Mackay, T.F.C., and R.R.H. Anholt 2006, Annu. Rev. Genomics Hum. Genet. 7: 339–67; McGettigan, J., R.K.J. McLennan, K.E. Broderick, L. Kean, A.K. Allan, P. Cabrero, M.R. Regulski, V.P. Pollock, G.W. Gould, S.-A. Davies, and J.A.T. Dow 2005, Insect Biochem. Mol. Biol. 35: 741–754; MedlinePlus 2007, http://www.nlm.nih.gov/medlineplus/spanish/ency/article/000464.htm; Modelska, K., and S. Cummings 2002, Clin. Endocrinol. Metab. 87: 6–23; St. John, M.A.R., and T. Xu 1997, Am. J. Hum. Gen. 61: 1006-1010; Terashima, J., K. Takaki, S. Sakurai, and M. Bownes 2005, J. of Endocrinology 187: 69-79; Yang, J., C. McCart, D.J. Woods, S. Terhzaz, K.G. Greenwood, R.H. Ffrench-Constant, and J.A.T. Dow 2007, Physiol. Genomics 30: 223-231.

A novel record of Drosophilidae species in the Cerrado biome of the state of Mato Grosso, west-central Brazil.

Blauth, Monica L.,[1] and Marco S. Gottschalk[2]. [1]Universidade do Estado de Mato Grosso (UNEMAT), *Campus* de Tangará da Serra, Departamento de Ciências Biológicas; [2]Universidade Federal do Rio Grande do Sul (UFRGS), Departamento de Genética, Laboratório de *Drosophila.*

Introduction

Systematic studies on the ecology and distribution of Drosophilidae species in Brazil started in the 1940's (Dobzhansky and Pavan, 1943, 1950; Pavan, 1959). From then on, research groups were formed to investigate Drosophilidae species in different localities in Brazil, especially in the south-east and Atlantic Forest. More recently, by the end of the 1990's, systematic studies were conducted also in west-central Brazil, more specifically in the Cerrado biome (Tidon *et al.*, 2003, 2005; Tidon, 2006), in southern Brazil, in the Atlantic Forest biome (De Toni and Hofmann, 1995; Schmitz *et al.*, 2007; Gottschalk *et al.*, in press), and in the north, in the Amazonian biome (Martins,