TDR SMALL GRANT APPLICATION FORM

Final Technical Report

By Elyes Zhioua, Pasteur Institute of Tunis, Tunisia

Proposal ID no: SGS 14/8

Project title Evaluation of rodent bait treated with fipronil for feed through and systemic control of *Phlebotomus papatasi* as preventive measures against zoonotic cutaneous leishmaniasis.
Abstract / Executive summary Zoonotic cutaneous leishmaniasis (ZCL) is a vector-borne disease caused by *Leishmania major* and transmitted by the bite of the phlebotomine sand fly *Phlebotomus papatasi*. ZCL is considered to be a neglected tropical disease, affecting about 1.5 million people per year. The disease is widespread in low-income countries of the Old World from Morocco to Afghanistan. Although ZCL is generally not fatal, the lesions may cause disfigurement and severe psychological and social distress to infected individuals. There is no effective vaccine against leishmaniasis and control of ZCL is based on measures directed against the sand fly vector and rodent reservoir of *L. major*. Indoor residual spraying is effective in reducing the incidence of ZCL, but requires expensive yearly application of insecticides and is thus not widely used in low-income countries. It also may adversely affect human health and the environment. In Tunisia, the incidence of ZCL per 100,000 inhabitants in semi-arid, arid, and Saharan bioclimatic zones is 0.1, 2.3, and 1.5, respectively. To date, neither a vector nor rodent control program is available in Tunisia. Therefore, this proposal aims to set up an integrated vector management program to control ZCL in endemic foci of Tunisia by evaluating new strategy that address the triangular components of this disease: vector, rodent reservoir, and human by validating a new approach based on the use of a systemic insecticide delivered as oral to rodent reservoir hosts to control populations of *P. papatasi* associated with rodent reservoirs, to reduce the infection rate of sand fly vector with *L. major*, and subsequently to reduce the incidence of ZCL. This program will be conducted in Tunisia for several reasons: (1) endemicity of disease (2) limited current control options (3) access to laboratory models and field sites for testing intervention strategies and (4) ability to conduct field-based research in a representative region that is general quite stable politically. Lessons learned will inform public health policies in Tunisia and in the Eastern Mediterranean nations.

Rationale and background

Zoonotic cutaneous leishmaniasis (ZCL) is a vector-borne disease caused by *Leishmania major* and transmitted by the bites of the phlebotomine sand fly *Phlebotomus papatasi* [1]. Fat sand rat (*Psammomys obesus*) and the desert's gird (*Meriones shawi*) are the main reservoirs of *L. major* in North Africa [2, 3]. Cutaneous leishmaniasis is considered by the WHO to be a neglected tropical disease, affecting about 1.5 million people per year [4]. The disease is widespread in low-income countries of the Old World from Morocco to Afghanistan where it constitutes a serious health problem [4]. Leishmaniasis is strongly correlated with poverty [5]. Although ZCL is generally not fatal, the lesions produced may cause substantial disfigurement and severe distress to infected individuals with lifelong psychological and social consequences [6].

To date there is no effective vaccine against leishmaniasis [7]. Control of ZCL is currently based on measures against the sand fly vector and rodent reservoir of *L. major*. Although indoor residual spraying (IRS) is effective in reducing the incidence of ZCL [8], it requires expensive yearly application of residual insecticides and its applicability is thus limited by financial constraints in low-income countries. It also may produce health problems in humans [9] and detrimental environmental effects. Its effectiveness may be reduced by the development of insecticide resistance in sand fly populations [10]. Insecticide-treated curtains or bednets (ITNs) offer effective protection against *P. papatasi*; but transmission continues as before after cessation of these measures [11]. Despite their efficacy in the interruption of ZCL transmission, programs based on the distribution of ITNs are poorly implemented in many endemic countries and are beyond the means of many families in ZCL-endemic villages [5]. Although poisoning ZCL rodent reservoirs with zinc phosphide has reduced the incidence of ZCL, this approach is ecologically unsound [12]. An alternate method such as zooprophylaxis using rabbits has also been explored as a means to reduce abundance of *P. papatasi* inside houses [13]. However, breeding rabbits in man-made underground holes located in the peri-doemestic area may lead to the establishment of sand fly breeding site and subsequently increase the risk factor for ZCL.

ZCL is endemic in Central and Southern Tunisia and it represents a serious public health problem [14-15]. In 1990, an outbreak of ZCL occurred in the governorate of Sidi Bouzid, Central Tunisia with 1618 cases mostly located in the town of Sidi Bouzid (42, 000 inhabitants). A field of chenopods surrounds the city of Sidi Bouzid. It is important to point out that the diet of *P. obesus* is limited only to chenopodes and therefore, this rodent species inhabits a complex burrows system associated with this chenopodiacae. A control method based on the destruction of the field of chenopods by mechanical plowing and planting trees up to a radius of 2 Km around the town of Sidi Bouzid, provided an efficacy in the prevention of the transmission of ZCL up to 91.5% [16]. Modification of the biotope of *P. obesus* was shown to be effective in controlling ZCL in Tunisia and in other endemic countries located in the Eastern Mediterranean region [16-17]. The Tunisian National Control of Parasitic Diseases Program instituted rodent surveillance of a radius of 2 Km around villages with more than 5000 inhabitants located in endemic areas [16]. Therefore, this approach has been recommended as a preventive measure against ZCL in the Eastern Mediterranean region (**see The work of WHO in the in the Eastern Mediterranean region, Annual report of the Regional Director Jan 1 through Dec 31, 2004, pp: 111**). However, it is of major epidemiological importance to point out that this approach is effective only in foci where *P. obesus* is the dominant rodent reservoir host species.

During the past 20 years, agricultural development took place around most of villages located in the governorate of Sidi Bouzid and subsequently has led to the destruction of the fat sand rat *P. obesus* habitat, resulting in the disappearance of this rodent species from around human settlements in many endemic parts of Tunisia [18-19]. The gerbil *M. shawi* has subsequently become the principal reservoir host of *L. major* in many of these areas [19]. *Meriones shawi* is nocturnal, feeds mostly on grain and fruit, and inhabits complex burrows systems associated with one of its main food resources, the jujube tree, *Ziziphus zizyphus*. In addition, the thorn of the tree preclude predator from accessing to burrows. These burrows have moderate, stable temperatures and elevated humidity which creates a suitable microclimate for the immature and adult stages of *P. papatasi*. Adult female *P. papatasi* also utilize *M. shawi* as their primary blood meal source, and sand fly larvae utilize the rodent's feces, as well as plant debris that accumulate inside the animals' burrows, as their main food source (Zhioua, unpublished). The agriculture development has lead to the establishment of *M. shawi* as the main rodent reservoir host species around human settlement [19]. Habitat modification based on the destruction of the jujube tree in order to destroy gird's burrows is not feasible. Deep plowing is not effective to remove the deep-grounded jujube tree. In addition, these trees play an important ecological role in maintaining the soil to prevent desertification. Therefore, alternative methods to control populations of *P. papatasi* associated with *M. shawi* are highly needed.

The closely linked relationship between *P. papatasi* and *M. shawi,* make insecticide application targeted at *M. shawi* an effective approach for controlling populations of *P. papatasi* associated with this reservoir host species. Identification of larval sand fly habitat is problematic, and even if identified many habitats such as rodent burrows are not easily accessible, so current *P.*

papatasi control measures only target adult sand flies. In recent years, insecticide treated rodent baits have been explored as a method to control insect vectors that feed on rodent hosts [20]. These treated baits have systemic and feed through insecticidal activity, which kills the blood feeding female, and the immature stages that feed on cattle feces [21]. Thus, linking adult sand flies with their larval diet is of major epidemiological importance [22]. Various insecticides such as imidacloprid [23] and fipronil [24] have been evaluated for use in rodent bait. It is important to point out that all these aforementioned studies were performed only in laboratory bioassays.

Objectives and outcomes

In the present study we aim to:

Objectives	Expected outcomes
1. Test the impact of a large field application using fipronil-treated rodent bait as a systemic and feed through insecticide for controlling populations of *P. papatasi* feeding on *M. shawi*.	We expect a significant reduction of the abundance of *P. papatasi* associated with *M. shawi* in the treated site compared to control site during the whole sand fly season
2. Assess the effect of fipronil-treated baits on the infection rates of *P. papatasi* with *L. major.*	We expect a significant reduction in the infection rate of *P. papatasi* with *L. major* in the treated site compared to the control site.
3. Assess the effect of fipronil-treated baits on the infection rates of *M. shawi* with *L. major*.	We expect a significant reduction in the infection rate of *M. shawi* with *L. major* in the treated site compared to the control site.

Design and methods

Preliminary data

Data from Zhioua's laboratory at Pasteur Institute of Tunis indicated that insecticide treated rodent baits are effective in controlling populations of *P. papatasi* that feed on rodent hosts under laboratory conditions. Such baits have systemic and feed through insecticidal activity, killing the blood feeding female as well as the immature stages that feed on the rodent feces. Our study is the first active field trial concerning the use systemic and feed-through insecticide delivered as oral bait to control sand fly populations associated with rodent reservoir. **However, from an epidemiological point of view, it is important to ascertain whether using systemic and feed-through insecticide delivered as oral bait to rodent reservoir will reduce the transmission of *L. major*.**

Hypothesis

We hypothesize that using fipronil-treated rodent bait to control *P. papatasi* that feed on rodent reservoirs will not only reduce abundance of *P. papatasi* associated with *M. shawi* , but it will reduce also the infection rates of *P. papatasi* with *L. major* , the infection rates of *M. shawi* with *L. major*, and subsequently the transmission of ZCL in endemic foci of Tunisia.

Experimental approach

1.Test the impact of using fipronil-treated bait as systemic and feed through insecticide for controlling populations of P. papatasi feeding on M. shawi

The field studies will be conducted at two sites located 2km apart in the eastern part of the town of Sidi Bouzid, Tunisia (34°51N, 9°29'E) from June to October 2015, a period corresponding the two main peaks of activity of *P. papatasi* [27]. These sites are known to harbour an important population of *M. shawi*. Each site will contain approximately 50 active rodent burrows. Two consecutive weeks prior to bait application, the number of sand flies per site will be evaluated by placing sticky traps at the entry of each burrow overnight. Sand flies will be removed from the sticky traps the next day with a fine-haired brush, counted, and identified to species level by using the identification keys of Croset et al. [28]. After the initial sand fly assessment, sites will be randomly assigned to receive the fipronil bait or the untreated bait. Following the initial sand fly assessment, a single application of treated bait (150 g) will be placed in front of each burrow, and will be repeated every 5 weeks. Bait will be made in house and consists to crushed barely mixed with technical grade fipronil at a concentration of 0.005%. Sand flies will be monitored in the treated and control sites by using sticky traps placed at the entry of rodent's burrows from June until the end of October 2015 on a weekly basis. The reduction in the number of *P. papatasi* collected in the treated area will be calculated using a modified Abbott's formula [29]:

% CONTROL/REDUCTION = $100-[(T_{after}/T_{before})/(U_{after}/U_{before}) \times 100]$

Where: T= number of *P. papatasi* in the treated area; U= number of *P. papatasi* in the untreated area. ANOVA or kruskal test will be used to test for difference between number of *P. papatasi* before and after treatment in the control as well as in the treated site.

2. *Assess the effect of fipronil-treated baits on the infection rates of P. papatasi with L. major*

Collected females *P. papatasi* from control and treated sites will be pooled based on trapping night and collecting site with a maximum of 30 individuals per pool and will be placed in 1.5 ml tubes and then will be stored at -80°C. Pools of sand flies will be examined for the presence of *L. major*. Extracted DNA will be amplified by nested PCR. Two-stage PCR will be carried out in two separate tubes [30-31]. The first stage PCR uses the forward primer IR1 (5' GCTGTAGGTGAACCTGCAGCAGCTGGATCATT 3', at the 3'end of the small subunit rRNA gene) and the reverse primer IR2 (5'GCGGGTAGTCCTGCCAAACACTCAGGTCTG 3', at the 5' end of the large subunit rRNA gene). The ITS1-5.8S fragment will be amplified using the nested forward primer ITS1F (5'GCAGCTGGATCATTTTCC 3'; overlapping the 3' end of the small subunit rRNA gene and ITS1) with the nested reverse primer ITS2R4 (5' ATATGCAGAAGAGAGGAGGC 3'; at the 5'end of ITS2) [30]. The first amplification reaction will be carried out in 20µl, containing 1xTaq polymerase buffer B (Invitrogen), 1.5 mM MgCl2, 60 µM each dNTP (Invitrogen), 1 µM primer IR1, 1 µM primer IR2, 1 unit Taq polymerase (Invitrogen) and 50 ng template DNA. The cycling conditions are an initial denaturation at 94°C for 3 min, followed by 37 cycles each consisting of three steps: denaturation 30s at 94°C; annealing 30s at 58°C and extension 90s at 72°C. After the last cycle, the extension step will be continued for a further 10 min. The nested amplification will be carried out in a second tube, with the reaction mix again totalling 20 µl and containing the same reagents as the first stage, except that the primers were now 1 µM primer ITS1F and 1µM primer ITS2R4, and the target DNA will be provided by adding 1 µl of the completed first-stage PCR reaction. The thermocycler program is as described for the first stage. Twenty microliters of the amplification products will be analyzed by 1.5% agarose gel and will be visualized under UV light. Positive samples will yield a PCR product of 462 bp. Contaminations by amplifications will be avoided by using drastic physical separation as well as decontamination procedure. Cross contamination will be monitored by

negative controls for sample extraction and PCR solutions. The final PCR products will be directly sequenced to identify *Leishmania* species (*L. major*) infecting *P. papatasi* and other sand fly species. A minimum infection rate of *P. papatasi* with *L. major* will be determined and compared between control and treated sites using ANOVA or equivalent non-parametric test.

3-Assess the effect of fipronil-treated rodent bait on the infection rates of *M. shawi* with *L. major*

In Tunisia, the infection rate of *M. shawi* with *L. major* increases over sand fly season and reach a peak of 53% during September-October [19] corresponding to the second main peak of *P. papatasi* [27]. Therefore, we will assess the effect of fipronil-treated rodent bait on the infection of *M. shawi* with *L. major* during November-December.

Sherman live traps, baited with dates, will be placed at the entrance of each active rodent burrow in both study sites for 4 consecutive nights per week during November and December 2015. Trapped rodents will be collected from the field and transported to the laboratory for examination. Trapped rodents will be identified, sexed, and examined for cutaneous lesions mainly at the tail and the ears. Clinical manifestation of *Leishmania* infection will be assessed by a thorough skin examination. Signs include depilation, hyper-pigmentation of the higher edge of the ear and the tail, infiltration, and dissemination of parasites with the presence of small nodules or partial destruction of organs.

All trapped rodents from each site will be anaesthetized by subcutaneous injection of 200 µl of ketamine (10 mg/ml) (Merial, Lyon, France) and biopsies will be taken from the ears and tails and will be examined for the presence of *L. major* by PCR as described previously. The infection rates of *M. shawi* with *L. major* from the treated and control sites will be compared by ANOVA or kruskal test.

In addition, the infectiousness of *M. shawi* to *P. papatasi* will be assessed by xenodiagnosis [32]. Each anaesthetized rodent from each site will be placed in a cage containing between 15 to 30 laboratory-colonized females and 10 males *P. papatasi* for 2 hrs. Engorged females *P. papatasi* will be transferred to clean cages containing sugar solution and will be allowed to digest blood for 5 days. After digestion, females *P. papatasi* will be pooled by rodent and will be examined for the presence of *L. major* by PCR as described previously. The infection rates of *P. papatasi* with *L. major* fed on rodents collected from treated and control sites will be compared by ANOVA or kurskal test.

Results (data collection)

Test the impact of using fipronil-treated bait as systemic and feed through insecticide for controlling populations of P. papatasi feeding on M. shawi

The field study is being conducted at two sites located 2km apart in the eastern part of the town of Sidi Bouzid, Tunisia (34°51N, 9°29'E) from June to October 2015, a period corresponding the two main peaks of activity of *P. papatasi* [27]. These sites were investigated during July 2015 for rodent activities. We found out that these two sites are harbouring an important population of *M. shawi*. Each site contains at least 60 active rodent burrows. For two consecutive weeks prior to bait application, the number of sand flies per site was evaluated by placing sticky traps at the entry of each burrow overnight. Sand flies were removed from the sticky traps the next day with a fine-haired brush, counted, and identified to species level by using the identification keys of Croset et al. [28]. The results of sand flies count during the pre-treatment period are show in the table below.

After this initial sand fly assessment, sites were randomly assigned to receive the fipronil bait or the untreated bait. Following this initial sand fly assessment, a single application of treated bait (150 g) was placed on September 1, 2015 in front of each burrow. Sand flies are being monitored in the treated and control sites by using sticky traps placed at the entry of rodent's burrows until the end of October 2015 on a weekly basis. The reduction in the number of sand flies collected in the treated area will be calculated using a modified Abbott's formula [29] at the end of the study period.

Following the treatment, the number of sand flies associated with rodent's burrows was reduced significantly (Table 1). To the contrary, in the control area, no reduction in the number of sand flies associated with rodent's burrows was observed (Table 1). It is of major epidemiological importance to point out that the reduction in sand flies observed at the end of the season is related to the seasonal activity.

The analysis of collected sand flies associated with rodent's burrows in the treated and in control for the presence of *L. major* areas is underway. Similar analysis is being performed concerning the prevalence of infection of *M. shawi* with *L. major* in the treated and control areas.

Table 1. Sampling of sandflies by sticky traps during pre-treatment period and post-treatment period.

Site of study	Date of field work	Number of investigated active rodents' burrows		Number of sandflies per area		Density of sandflies (number of sanflies per $m^2$of sticky traps)	
		Treated area	Control area	Treated area	Control area	Treated area	Control area
Governorate of Sidi Bouzid	18/08/2015	60	60	61	35	14	8
	25/08/2015	60	60	130	121	29	27
	01/09/2015	70	70	204	123	38	23
	02/09/2015	**Treatment of the treated area with the bait containing fipronil**					
	08/09/2015	Climatic factor , rain					
	16/09/2015	88	88	66	176	10	26
	21/09/2015	65	65	31	44	7	9
	29/09/2015	76	76	44	50	8	9
	07/10/2015	Climatic factor , rain					
	20/10/2015	63	63	22	19	5	4
	27/10/2015	60	60	7	3	2	1

List bibliographic references.

1.Ben Ismail, R., Gramiccia, M., Gradoni, L., Helal, H., Ben Rachid, M.S., 1987. Isolation of *Leishmania major* from *Phlebotomus papatasi* in Tunisia. Trans. Roy. Soc. Trop. Med. Hyg. 81: 749.

2. Fichet-Calvet, E., Jomâa, I., Ben Ismail, R., Asford, R.W., 2003. *Leishmania major* infection in the fat sand rat *Psammomys obesus* in Tunisia: interaction of hosts and parasite populations. Ann. Trop. Med. Parasitol. 97: 593-603.

3.Rioux, J.A., Petter, F., Zahaf, A., Lanotte, G., Houin, R. 1986. Isolement de *Leishmania major* Yakimoff et Schokhor, 1914 [Kinetoplastida-Trypanosomidae] chez *Meriones shawi shawi* (Duvorney, 1842) [Rodentia-Gerbillidae] en Tunisie. Ann. Parasitolo. Hum. Comp. 6: 139-145.

4.Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., WHO Leishmaniasis Control Team, 2012. Leishmaniasis worldwide and global estimates of its incidence. Plos One, 7(5): e35671. doi: 10.1371/journal.pone.0035671.

5. Alvar, J., Yactayo, S., Bern, C., 2006. Leishmaniasis and poverty. Trends Parasitol. 12: 552-557.

6.Kassi, M., Afghan, A.K., Rehman, R., Kazi, P.M., 2008. Marring leishmaniasis: The stigmatization and the impact of cutaneous leishmaniasis in Pakistan and Afghanistan. PLOS Negl Trop. Dis. 2: doi:10.1371/journal.pntd.0000259.g001.

7.Ben Hadj Ahmed, S., Chelbi, I., Kaabi, B., Cherni, S., Derbali, M., Zhioua, E., 2010. Differences in the salivary effects of wild-caught vs. colonized *Phlebotomus papatasi* Scopoli (Diptera: Psychodidae) on the development of zoonotic cutaneous leishmaniasis in BALB/c Mice. J. Med. Entomol. 47: 74-79.

8.Benzerroug, E.H., Benhabylles, N., Izri, M.A., Belhacene E.K., 1992. Les pulvérisations intra et péri-domicilaires de DDT dans la lutte contre la leishmaniose cutanée zoonotique en Algérie. Ann. Soc. Belge Méd. Trop. 72: 5-12.

9.Hernández, A.F., R Alarcón, R., 2011. Pesticides and asthma. Curr Opin Allergy Clin Immunol. 11: 90-96.

10.Alexander; B, Barros, V.C., de Souza, S.F., Barros, S.S., Teodoro, L.P., Soares, Z.R., NF Gontijo, N.F., Reithinger, R., 2009. Susceptibility to chemical insecticides of two Brazilian populations of the visceral leishmaniasis vector *Lutzomyia longipalpis* (Diptera: Psychodidae). Trop. Med. Int. Health 14: 1272-1277.

11.Jalouk, L., Al Ahmed, M., Gradoni, L., Maroli, M., 2007. Insecticide-treated bednets to prevent anthroponotic cutaneous leishmaniasis in Aleppo governorate, Syria: results from two trials. Trans. R. Soc. Trop. Med. Hyg. 101: 360-367.

12.Veysi, A., Vatabdoost, H., Yaghoobi-Ershad,i M.R., Arandian, M.H., Jafari, R., Hosseini, M., Abdoli, H., Rassi, Y., Heidari, K., Sadjadi, A., Fadaei, R., Ramazanpour, J., Aminian, K., Shirzadi, M.R., Akhavan, A.A., 2012. Comparative study on the effectiveness of coumavec® and zinc phosphide in controlling zoonotic cutaneous leishmanisis in a hyperendemic focus in Central Iran. J. Arthropod-Borne Dis. 6: 18-27.

13.Chelbi, I., Kaabi B., Derbali M., Ben Hadj Ahmed S., Dellagi, K., Zhioua, E., 2008 Zooprophylaxis: impact of breeding rabbits around houses on reducing the indoor abundance of *Phlebotomus papatasi.* Vector-Borne Zoo. Dis. 8: 741-747.

14.Ben Salah, A., Kamarianakis, Y., Chilf, S., Ben Alya, N., Prastacos, P., 2007. Zoonotic cutenous leishmaniasis in Central Tunisia. Inter. J. Epidemiol. 36 : 991-1000.

15.Chelbi, I., Kaabi, B., Béjaoui, M., Derbali, M., Zhioua, E., 2009. Spatial correlation between *Phlebotomus papatasi* Scopoli (Diptera: Psychodidae) and incidence of zoonotic cutaneous leishmaniasis in Tunisia. J. Med. Entomol. 46: 400-402.

16.Ben Salah, A., Ben Alya, N., Zaâtour, A., Garraoui, A., Dellagi, K., Ben Ismail, R., 2005. Long-term evaluation of the efficacy of environmental changes as a control strategy of zoonotic cutaneous leishmaniosis. Proceeding of the third world congress on leishmaniosis, Palermo, Italy, pp: 114.

17.Kamhawi, S., 1993. Environmental changes in the control of zoonotic cutaneous leishmaniasis focus. Arch. Institut Pasteur Tunis 70: 383-390.

18.Ben Salah, A., Kamarianakis Y., Chlif .S, Ben Alya N., Prastacos P., 2007. Zoonotic cutaneous leishmaniasis in central Tunisia: spatio-temporal dynamics. Inter. J. Epidemiol. 36: 991-1000.

19.Ghawar, W., Toumi, A., Snoussi, M.A., Chlif, S., Zâatour, A., Boukthir, A., Bel Hadj Hamida, N., Chemkhi, J., Diouani, M.F., Ben Salah, A., 2011. *Leishmania major* infection among *Psammomys obesus* and *Meriones Shawi*: reservoirs of zoonotic cutaneous leishmaniasis in Sidi Bouzid (Central Tunisia). Vector-Borne Zoo. Dis. 11: 1561-1568.

20.Brochert, J.N., Davis, R.M., Poché, R.M., 2009. Field efficacy of rodent bait containing the systemic insecticide imidacloprid against the fleas of California ground squirrels. J. Vect. Ecol.34: 92-98.

21.Poché, R.M., Garlapati, R., Singh, M.I., Poché, D., 2013. Evaluation of fipronil oral dosing to cattle for control of adult and larval sand flies under controlled conditions. J. Med. Entomol. 50:833-837.

22.Mascari, T.M., Hanafi, H.A., Jackson, R.E., Ouhabi, S., Ameur, B., Faraj, C., Obenauer P.J., Diclaro, J.W., Foil, L.D., 2013. Ecological and control techniques for sand flies (Diptera : Psychodidae) associated with rodent reservoirs of leishmaniasis. Plos Neg Trop Dis 2013b; 7: e2434. Doi:10.1371/journal.pntd.0002434.

23.Wasserberg, G., Poché, R., Miller, D., Chenault, M., Zollner, G., Rowton, E., 2011. Imidacloprid as a potential agent for the systematic control of sand flies. J. Vect. Ecol. 36 (Suppl 1): S148-S156.

24.Ingenloff, K., Garlapati, R., Poché, D., Singh, M.I., Remmers, J.L., Poché, R.M., 2012. Feed-through insecticides for the control of the sand fly *Phlebotomus argentipes*. Med. Vet. Entomol. doi: 10.1111/j.1365-2915.

27.Chelbi, I., Derbali M., AL-Ahmadi, Z., Zaafouri, B., El Fahem, A., Zhioua, E., 2007. Phenology of *Phlebotomus papatasi* (Diptera: Psychodidae) relative to the seasonal prevalence of zoonotic cutaneous leishmaniasis in Central Tunisia. J. Med. Entomol. 44: 385-388.

28.Croset, H., Rioux, J.A., Master, M., Bayar, N., 1978. Les phlébotomes de la Tunisie (Diptera: Phlebotominae). Mise au point systématique, chrologique et éthologique. Ann. Parasitol. Hum. Comp. 53 :711-749.

29.Mount, G.A., 1981. *Amblyomma americanum*: area control of overwintered nymphs and adults in Oklahoma with acaricides. J. Econ. Entomol, 74: 24-26.

30.Parvizi. P, Mauricio. L, Aransay. A.M, Miles. M.A, Ready. P.D, 2005. First detection of *Leishmania major* in peridomestic Iranian sandflies: comparison of nested PCR of nuclear ITS ribosomal DNA and semi nested PCR of minicircle kinetoplast DNA. Acta Trop. 2005; 93: 75-83.

31.Parvizi P., Ready P.D. Nested PCRs of nuclear ITS-rDNA fragment detect three *Leishmania* species of gerbils in sandflies from Iranian foci of zoonotic cutaneous leishmaniasis. Trop. Med. & Intern. Health 2008; 13: 1159-1171.

32. Derbali, M., Chelbi, I., Ben Hadj Ahmed, S., Zhioua, E., 2012. *Leishmania major* Yakimoff et Schokhor, 1914 (Kinetoplastidae: Trypanosomatidae) in *Meriones Shawi* Duvernoy, 1842 (Rodentia: Gerbillidae): persistance of the infection in merions and its infectivity for the sand fly vector *Phlebotomus (Phlebotomus) papatasi*, Scopoli, 1786 (Diptera: Psychodidae). Bull. Soc. Pathol. Exot. 105: 399-402.

Enter text here