Survival of the Immature Stages of the Malaria Vectors *Anopheles pseudopunctipennis* and *Anopheles argyritarsis* (Diptera: Culicidae) in Northwestern Argentina

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Abstract

In order to optimally time the application of control measures to reduce populations of malaria vectors, program managers need to know precisely when the vulnerable larval stage will be most abundant at each specific breeding site. Therefore at 4 different breeding sites of the malaria vectors, *Anopheles pseudopunctipennis* Theobald and *Anopheles argyritarsis* Robineau-Desvoidy in northwestern Argentina, we recorded the calendar dates during spring and summer when different life stages appeared, and in each of these 2 seasons, we measured the duration of each life stage and the probability that it would transition to the subsequent stage or die. Larval samples were collected during the spring and summer of 2008-2009 at 4 localities in northwestern Argentina. These larvae were reared individually in plastic containers in which the volume of water was kept constant, temperature and photoperiod were controlled, and a standard amount of food was provided each day. The data were analyzed by multistate models, a nonparametric model of survival without covariates, a survival model with covariates, a Cox-type survival model with specific co-variates, and models of reduced rank. We collected 1,643 larvae of which 1,404 reached adulthood. Of these 1,119 were *An. pseudopunctipennis*, and 285 were *An. argyritarsis*. Both *An. pseudopunctipennis* and *An. argyritarsis* were abundant in autumn (55.3% and 66.7%, respectively). Considerably more individuals transitioned from larvae to pupae than from pupae to adults. The probability of an individual remaining in the larval stage for the first 2 days was close to 100% and then decreased. The transition from the larval stage to death was significant in the summer**.** The breeding site at Rosario de la Frontera exhibited a particularly significant effect on the transition from the larval stage to death, i.e., greatly increased larval mortality. The results obtained in the present study are substantial contributions to the bionomics of *An. pseudopunctipennis* and *An. argyritarsis*. According to our results, mosquito source management programs should be focused on the larval stage during the summer season and principally at Rosario de la Frontera River. These actions could substantially reduce the production of the adult vectors and potentially reduce transmission of malaria in northwestern Argentina.

Key Words: immature stages, *Anopheles pseudopunctipennis*, *Anopheles argyritarsis*, multistate models, survival, northwestern Argentina

Resumen

Para optimizar el tiempo de aplicación de medidas tendientes a reducir las poblaciones de vectores de la malaria, los directores de programas necesitan saber con precisión cuando el estado larval vulnerable será más abundante en cada sitio específico de cría. Por lo tanto, en 4 diferentes sitios de cría de los vectores de la malaria, *Anopheles pseudopunctipennis* Theobald y *Anopheles argyritarsis* Robineau - Desvoidy, en el noroeste de Argentina, registramos las fechas durante la primavera y el verano cuando los diferentes estados de vida aparecieron, y en cada una de estas 2 estaciones climáticas, medimos la duración de cada estado de vida y la probabilidad que había para la transición al siguiente estado o para morir. Se recogieron muestras de larvas durante la primavera y el verano de 2008-2009 en 4 localidades en el noroeste de Argentina. Estas larvas fueron criadas individualmente en envases de plástico en la que el volumen de agua se mantuvo constante, la temperatura y el fotoperíodo se controlaron, y una cantidad estándar de la comida se proporcionó cada

día. Los datos se analizaron mediante modelos multiestados, un modelo no paramétrico de supervivencia sin co-variables, un modelo de supervivencia con co-variables, un modelo de supervivencia de tipo Cox con co-variables específicas y un modelo de rango reducido. Se recolectaron 1.643 larvas, de las cuales 1.404 llegaron a las formas adultas, identificándose 1.119 como *An. pseudopunctipennis* y 285 como *An. argyritarsis*. Tanto *Anopheles pseudopunctipennis* como *An. argyritarsis* fueron abundantes en el otoño (55,32% y 66,67%, respectivamente). Considerablemente más individuos pasaron de ser larvas a pupas que de pupas a adultos. La probabilidad de un individuo de permanecer en el estado larval en los primeros 2 días fue cercana al 100%, disminuyendo luego. La transición de larva a muerte fue significativa en el verano. El sitio de cría de Rosario de la Frontera exhibió un efecto significativo en la transición del estado larval a la muerte, es decir, aumentó en gran medida la mortalidad de las larvas. Los resultados obtenidos en el presente estudio son importantes contribuciones a la bionomía de *An. pseudopunctipennis* y *An. argyritarsis*. Según nuestros resultados, los programas de manejo de criaderos de mosquitos deben centrarse en el estado larval durante el verano y principalmente en el río Rosario de la Frontera. Estas acciones podrían reducir sustancialmente la producción de los vectores adultos y potencialmente reducir la transmisión de la malaria en el noroeste de Argentina.

Palabras Clave: estados inmaduros, *Anopheles pseudopunctipennis*, *Anopheles argyritarsis*, modelos multiestados, supervivencia, Noroeste de Argentina

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Malaria remains one of the most important tropical infectious diseases that affect people worldwide. In fact, 219 million cases of malaria and 660,000 deaths were reported in 2010 (Paaijmans et al. 2007; World Health Organization 2012). In the Americas, at least 30% of the population of the 21 countries is living at some degree of risk, and approximately 8% of the population is at high risk. Argentina, El Salvador, Mexico, and Paraguay remain in the pre-elimination phase of malaria eradication (World Health Organization 2012).

The number of confirmed cases in the Americas decreased from 1.18 million in 2000 to 490.000 in 2011 (World Health Organization 2012). Brazil and Colombia accounted for 68% of the cases in 2011. Reductions in the number of confirmed cases and in the case incidence rates of more than 75% were recorded in 13 countries (Argentina, Belize, Bolivia, Costa Rica, Ecuador, El Salvador, French Guiana, Guatemala, Honduras, Mexico, Nicaragua, Paraguay, and Suriname) between 2000 and 2011 (World Health Organization 2012).

Anopheles pseudopunctipennis Theobald (Diptera: Culicidae) is the primary mosquito incriminated in malaria transmission in the foothills of South America, including Argentina (Zimmerman 1992). The larval habitats of this species along their wide geographical distribution have been characterized as freshwater stream pools that are partially shaded and contain clean water and filamentous green algae. The few studies that have focused on the biological and ecological aspects of immature forms of *An. pseudopunctipennis* reported that larvae could also be found in artificial containers (tanks, fountains, rice paddies, and marshy meadows) and that environmental variables, including filamentous green algae, altitude, shallow water, annual rainfall, and water temperature, influence larval abundance (Shannon 1930; Hoffmann 1931; Hoffmann & Samano 1938; Rozeboom 1941; Hackett 1945; Downs et al. 1948; Savage et al. 1990; Rejmankova et al. 1991; Fernández-Salas et al. 1994; Berti et al. 1993; Manguin et al. 1996; Grillet 2000; Dantur Juri et al. in press).

Since the beginning of the 20th century, *An. pseudopunctipennis* was reported as the main vector of malaria in northwestern Argentina, and this pioneering work was based on the characterization of the larval habitats of the species during different seasons (Shannon & Davis 1927). The densities of the immature stages of *An. pseudopunctipennis* have been shown to fluctuate from the end of the spring season to the autumn season, when malaria cases occur (Dantur Juri et al. in press).

In general, the availability of aquatic habitats depends on precipitation (Koenraadt et al. 2004; Fillinger et al. 2004; Paaijmans et al. 2007); however, the mean minimum and maximum air temperatures and the water temperature are also climatic variables that can exert influence on the abundance of *An. pseudopunctipennis* larvae in northwestern Argentina (Dantur Juri et al. in press).

Rates of larval and pupal mortality are affected by climatological factors, such as temperature (Bayoh & Lindsay 2003; Bayoh & Lindsay 2004; Paaijmans et al. 2007). Also precipitation can affect larval population dynamics through the flooding of habitats and the consequent flushing out of larvae (Edillo et al. 2004; Mutuku et al. 2006; Paaijmans et al. 2007).

Mortality of the immatures during development can be very high. Several studies have reported that only a small fraction (2-8%) of the hatched larvae survive to eventually become adults, and they attributed this high but variable mortality to the presence or absence of predators,

parasites, pathogens (Aniedu et al. 1993; Paaij-mans et al. 2007), or cannibalism (Okogun 2005; Paaijmans et al. 2007), among other biological factors.

The scarcity of larval resources (food/ space) results in longer development time and reductions in success and size at metamorphosis (Hawley 1985; Bradshaw & Holzapfel 1992; Renshaw et al. 1993; Maciá 2009). In adults, body size, survival, fecundity, mating success and flight capacity are lowered by scare or inadequate larval resources (Wada 1965; Steinwascher 1982; Reisen et al. 1984; Fisher et al. 1990; Maciá 2009).

To date, no study has quantified the effect of season of the year and local habitat on the development times of the different stages, nor the relationship between season and local habitat both on the duration of each life stage and the probability of transitioning to the subsequent life stage in populations of the malaria vectors, *Anopheles pseudopunctipennis* and *Anopheles argyritarsis,* in northwestern Argentina. We sought to meet these objectives in this study.

MATERIALS AND METHODS

Study Area

The subtropical mountainous rainforest called "Yungas" extends in northwestern Argentina from the Bolivian border (23°S) to the northern area of Catamarca province (29°S), and passes through Salta, Jujuy, and Tucumán provinces (Brown & Grau 1995; Brown et al. 2001).

The mean annual rainfall is 734 mm, and ranges from 700 to 1,000 mm. In northwestern Argentina the year can be divided into 3 seasons: a warm and dry spring (Sep-Dec), a warm and rainy summer (Jan-Apr), and a temperate and clammy fall-winter season (May-Aug) (Brown & Grau 1995).

All of the localities are located on the elevated floor of the piedmont of the subtropical mountainous rainforest, which consists of ecotonal areas with the Chaco xerophytic forest (Cabrera 1976; Brown & Grau 1995). The native vegetation—with the exception of the areas near roads and stream banks where it becomes less dense—is closed arboreal vegetation (Digilio & Legname 1966; Cabrera 1976; Dantur Juri et al. 2010a, 2010b). The canopy trees include *Blepharocalyx salicifolius* (H. B. K.) Berg. (Myrtaceae), *Enterolobium contorsiliquum* (Vell.) Morong, (Fabaceae) and *Juglans australis* Griseb. (Juglandaceae). The climber species belong to the Bignoniaceae, Ulmaceae, and Amaranthaceae. There are also vascular epiphytes, such as Bromeliaceae. *Tipuana tipu* (Benth.) Kuntze, *Jacaranda mimosifolia* D. Don. (Bignoniaceae), *Tabebuia avellanedae* Lor. ex Griseb. (Bignoniaceae), *Tecoma stans* (L.) C. Juss. ex Kunth (Bignoniaceae), and *Salix hum-*

boldtiana Willd. (Salicaceae) are found in clearings. The piedmont flora and fauna have suffered from anthropic pressure, which has led to its impoverishment and nearly complete destruction. Anthropogenic impacts in these areas originate from large sugarcane, soya, and citrus fields (Prado 1995; Dantur Juri et al. 2010a, 2010b).

Sampling Sites

Immature forms were sampled in various bodies of water (habitats) in which, according to the literature, immature anophelines have previously known to be present (Mühlens et al. 1925; Shannon & Davis 1927). Rosario de la Frontera River (S 25° 48' W 64° 58', 791 m asl) is located in Salta Province, and the Vipos River habitat (S 26° 29' W 65° 21', 786 m asl), El Cadillal Dam (S 26° 42' W 65° 16', 683 m asl), and Potrero de las Tablas River (S 26° 21' W 65° 21', 950 m asl) are situated in Tucumán Province. These breeding sites were sampled repeatedly across seasons, and they have characteristics of permanent of *Anopheles* larval habitats, such as fresh water and the presence of green algae.

Collection of *Anopheles* Immatures and Laboratory Rearing

Samplings were conducted seasonally during the period of 2008 to 2009 to register fluctuations of the populations of the different species during the different seasons (summer, fall, winter, and spring). The temperature of each body of water was determined using a digital thermo-hygrometer (Springfield Precision Instruments Inc., Wood Ridge, New Jersey, USA), and the pH was determined using reactive strips. The immature forms were collected in bodies of water connected to the rivers and to the dam using standard larval dippers (350 mL) (Service 1976; Fernández-Salas et al. 1994; Dantur Juri et al. in press). Twenty dips per site per sampling event were taken along the edge of each body of water.

The first instars were transported to the laboratory and placed 1 larva per 20 × 10.5 × 8 cm plastic container with demineralized water. They were reared to adults in an insectary that was maintained at 24 ± 2 °C. Each larva was fed with 0.2 mg of fish food per day until it either pupated or died. Water was added as necessary to compensate for evaporation. Rearing containers were checked daily and dead larvae or pupae were removed. Age of the individual at pupation was recorded as well as the time to reach adulthood. After the emergence, adults were sacrificed by exposure a low temperature, which preserved their appearance. These specimens were then placed in plastic vials until further identification by the taxonomic key in Forattini (2002). The exuvia of each larvae and pupae was collected from the vi-

als and preserved in 70% alcohol in appropriately labelled Eppendorf tubes.

Data Analysis

At first, the total abundance and the percentage of immature forms (larvae and pupae) of *An. pseudopunctipennis* and *An. argyritarsis* in relation to the each season and sampling site were calculated. Next, the data were analyzed by multistate models, which are suitable for the analysis of phenomena in which the individuals under study may be at different stages during the observation period. These models allow the estimation of the probability of an individual passing from one stage to another at each instant in time and the effect of certain factors on these probabilities (Meira-Machado et al. 2009). We defined the stages and transitions between stages. In this case, the stages considered were the following: larva (stage 1), pupa (stage 2), adult (stage 3), and death (stage 4). The possible transitions considered were the following: from larva to pupa (T1), from larva to death (T2), from pupa to adult (T3), and from pupa to death (T4) (Fig. 1). The adult and death stages were considered absorbing stages, i.e., the individuals were no longer monitored after reaching these stages.

The number and proportion of individuals who performed each transition were calculated. A nonparametric model of survival without covariates was used to estimate the cumulative risk at each transition over time and the probability of staying at each stage at each instance of time.

A survival model with covariates (semi-parametric model) was then used to estimate the effect of the climatic seasons and the local habitats on overall survival. This model was as follows:

$$h\,(t\,|\,Z) = H\,(t)\,\exp\,(\beta^{T}\;Z)$$

To determine whether the covariates have different effects depending on the transition, a Cox-type survival model was used (Cox 1972; Cox & Hinkley 1974;

Fan and Li 2002; Zeger et al. 2004), which considered specific covariates for each transition depending on the effect of the climatic season and

Fig. 1. Diagram that represents the stages and the transitions matrix.

of the local habitat on the transitions. This was modeled as follows:

$$h_{fg}\,(t\,|\,Z) = h_{fg,0}\,(t)\,\exp\,(\beta^{T}\;Z_{fg}),\text{ where}$$

h_{fg} = instantaneous risk at time t given covariates Z
$h_{fg,0}$ = instantaneous baseline risk (hazard) at time t
b = model coefficients.

The data were analyzed using models of reduced rank to estimate a lower number of parameters and thus to obtain more efficient estimators of the effects of each covariate at each transition (Reinsel & Velu 1998; Yuan & Lin 2006). This model was the following:

$$h_{k}\,(t\,|\,Z) = h_{k,0}\,(t)\,\exp\,(Y_{k}\alpha^{T}Z_{fg}),\text{ where}$$

k = index of transitions 1, 2, 3, and 4.

In addition, a measures the overall effect of each covariate on the risk of each of the transitions and γ_{k} represents the intensity of the effect on each transition. The αZ factor is considered to be a prediction score for individuals with covariates Z and determines the probability that a specific individual experienced an event. The parameter γ_{k} determines the size of the effect of the prediction score in transition k.

Results

A total of 1,643 larvae was collected of which 1,404 reached the adult stage. The most abundant species was *An. pseudopunctipennis* with 1,119 individuals, and only 285 of the individuals were identified as *An. argyritarsis*. The relative abundance of the larvae was high during autumn and spring, which was expected because *An. pseudopunctipennis* is the most abundant species during these seasons (55.32% and 33.24%, respectively). *Anopheles argyritarsis* was also equally abundant in the autumn and spring seasons (66.67% and 31.23%, respectively). During the winter, no immature form of either species was collected (Table 1).

The comparison of the abundance of both species by the sampling site (habitat) revealed that *An. pseudopunctipennis* was the dominant species (35.1% of the population) in El Cadillal and that *An. argyritarsis* was the most abundant species (51.2%) in Potrero de las Tablas (Table 2).

The number of individuals that made the transitions from larva to pupa (T1) was higher than the number that made the transition from pupa to adult (T2) (Fig. 2). The analysis of the percentage of individuals that made one transition revealed that 83% of the individuals made the transition from larva to pupa (T1) and that 88% of these individuals made the transition from pupa to adult (T3). In addition, a higher proportion of individuals died as larvae (T2, 17%) than as pupae (T4, 12%) (Fig. 3).
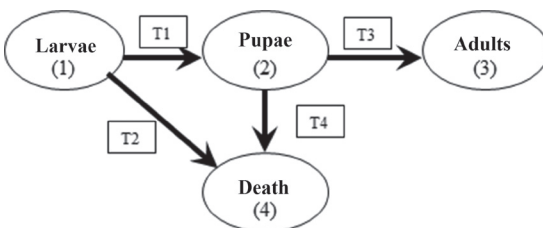
TABLE 1. TOTAL NUMBER OF INDIVIDUAL LARVAE (*N*) OF *ANOPHELES PSEUDOPUNCTIPENNIS* AND *AN. ARGYRITARSIS* COLLECTED DURING THE FOUR SEASONS OF 2008-2009.

Climatic seasons	N	%	N	%	N	%
Autumn	619	55.32	190	66.67	809	57.62
Spring	372	33.24	89	31.23	461	32.83
Summer	128	11.44	6	2.11	134	9.54
Winter	0	0	0	0	0	0
Total	1,119	100	285	100	1,404	100

The non-parametric model of survival without co-variables was used to determine the accumulated risk for each transition (T1, T2, T3 and T4) at each day. We found that doubling the time (days) increased the cumulative risk, meaning that in the case of the transition from larva to pupa (T1), the risk increases from the first to the second day (from 0 to 0.07). This risk remains relatively unchanged from the second to the fourth day, increases again (from 0.09 to 0.41) from the fourth to the eighth day, significantly increases (from 0.43 to 1.18) from the eighth to the 16th day (1.43 to 2.42), and continues to increase nearly to the end (2.42 to 3.42). In the case of T2, which is the transition from larva to death, the risk was low, i.e., there were few dead larvae. The transition from pupa to adult (T3) exhibited a higher risk than that associated with T1, and T4 (from pupa to death) exhibited a higher risk than that associated with T2 (Table 3).

As shown Fig. 4, the transition from larvae to pupae increased at days 4, 8, 16, and 20, and a similar, albeit higher, behavior was observed for the transition from pupae to adults. In contrast, the transition from larvae to death increased on day 8, but few dead larvae were observed. In addition, the transition from pupae to death doubled within 2 days, increased on day 4, and always exhibited a higher risk than that associated with T2 (Fig. 4).

Using the same model, the probabilities of being alive in the larval, pupal, and adult stages, and death were analyzed (Table 4, Fig. 5). The probability of being in the larval stage was close to 100% until the second day, decreased to 50% by the fourth day, and continued to decrease to 10% by the eighth day. It was found that the probability of being in the pupal stage was close to 0% for the first 3 days, increased on the fourth day to 26%, continued to increase during the next 2 wk to reach 40%, and then began to decrease. The probability of being in the adult stage was low initially, increased to 30% on the eighth day, increased to 67% at 2 wk, and remained high thereafter (Table 4, Fig. 5).

Using the survival model with co-variables, and firstly considering the climatic seasons as a co-variable, it was found that neither the spring nor the summer was significant (p = 0.78 and p = 0.56, respectively). However, the climatic seasons and the local habitats were considered together as co-variables, the seasons were found to be significant in Rosario de la Frontera River and Potrero de las Tablas River (p = 0.01 and p = 0.02, respectively).

The Cox survival model with co-variables (jointly considering the climatic seasons and the localities) that are specific to each transition (T1, T2, T3, and T4) showed that the transitions from larvae to death (T2) and from pupae to death (T4) are significant in the summer (p = 0.003 and p = 0.048, respectively). The transition from larvae to death (T2) decreased during the summer (p = 0.003), i.e., a smaller number of larvae died, and the same finding was observed with T4 in relation to the pupal stage (Table 5).

TABLE 2. TOTAL NUMBER (*N*) AND PERCENTAGE (%) OF LARVAE OF *AN. PSEUDOPUNCTIPENNIS* AND *AN. ARGYRITARSIS* COLLECTED AT DIFFERENT SAMPLING SITES DURING 2008-2009.

Sampling Sites*	*An. pseudopunctipennis*		*An. argyritarsis*		Total both species	
	N	%	N	%	N	%
RF	309	27.6%	30	10.5%	339	24.1%
VP	128	11.4%	35	12.3%	163	11.6%
EC	392	35.1%	74	26.0%	466	33.2%
PT	290	25.9%	146	51.2%	436	31.1%
Total	1,119	100%	285	100%	1,404	100%

*RF, Rosario de la Frontera River in Salta province; and the following in Tucumán province: VP, Vipos River; EC, El Cadillal; and PT, Potrero de las Tablas River.
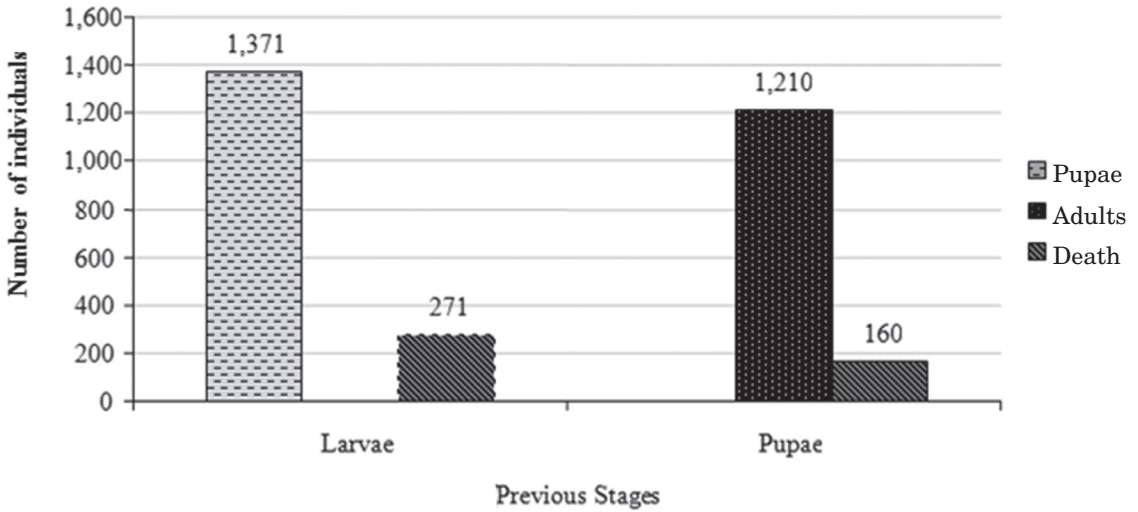
Fig. 2. Number of individuals that underwent a transition from one stage to another (larval, pupal, adult, and death).

The analysis of the effect of the local habitats on the transitions to death revealed that, in Rosario de la Frontera River, Potrero de las Tablas River, and Vipos River, there was a significant effect of the habitat on the transition from larvae to death (T2; $p = 0.050$, $p = 0.042$, and $p = 0.000$, respectively). Taking into account the value and sign of the coefficients, the risk of T2 is higher at Vipos River compared with the rest of the localities. Lastly, T4 (transition from pupae to death) was found to be significant only in Vipos River ($p = 0.020$; Table 6).

Lastly, the reduced rank model, in which the overall effect of each climatic season and locali-

ties (coefficient a) and the intensity of this effect in each transition (coefficient g) were estimated separately, showed that the major effects were achieved in the summer and in the Vipos River (a = -0.41932767 and a = 0.84045516, respectively). Higher values of the parameter g corresponded to the transitions from larvae to death (T2) and from pupae to death (T4; g = 0.7109526 and g = 0.8543723, respectively). The product of each estimated parameter a and g gave the effects of the co-variables on each transition. Thus, the effects of the climatic seasons on T2 and T4 (larvae to death and pupae to death) are higher in the summer than the spring, indicating the decreased risk
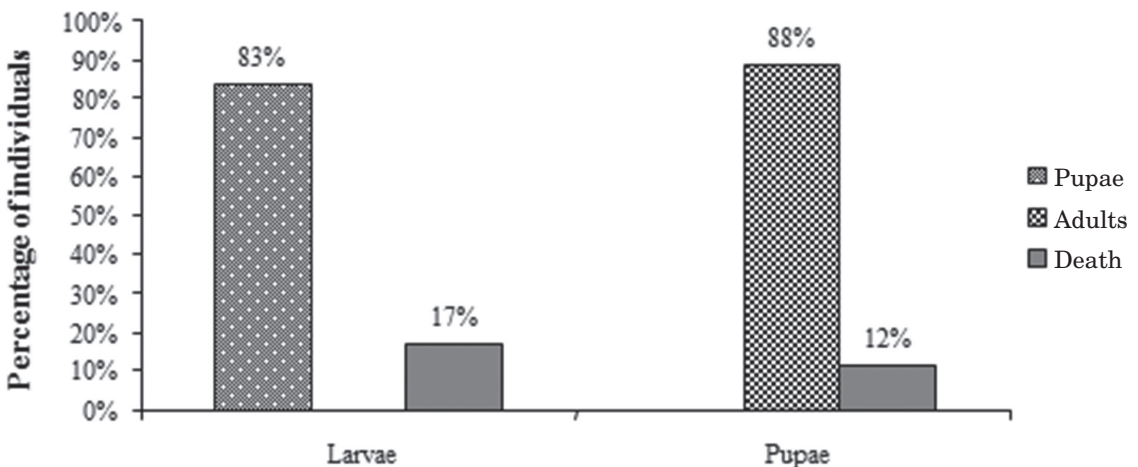

Fig. 3. The percentage of individuals that underwent a transition from one the stage to another (larvae, pupae, adult, and death).

TABLE 3. ACCUMULATED RISK FOR EACH TRANSITION (T1, T2, T3, AND T4) OVER TIME.

Time (days)	Accumulated risk (T1)	Accumulated risk (T2)	Accumulated risk (T3)	Accumulated risk (T4)
1	0	0	0	0
1.5	0	0	0	0
2	0.0791	0.10491	0.166667	0.83333333
3	0.0821	0.10491	0.166667	0.83333333
3.5	0.094	0.10491	0.166667	0.83333333
4	0.411	0.13517	0.838200	1.11800487
7	0.4226	0.13517	0.838200	1.11800487
7.5	0.4366	0.13517	0.838200	1.11800487
8	1.1857	0.20651	1.712920	1.2365731
9	1.1857	0.20651	1.714500	1.23815288
15	1.2718	0.20651	1.716084	1.23815288
15.5	1.4385	0.20651	1.716084	1.23815288
16	2.4298	0.20651	2.632000	1.32223696
20	3.4298	0.20651	2.632000	1.32223696
21	3.4298	0.20651	3.623228	1.33100889

The transitions were the following: from larva to pupa (T1), from larva to death (T2), from pupa to adult (T3), and from pupa to death (T4).

of the death of larvae and pupae in the summer (negative sign in the product). In contrast, the effect of the local habitat on T2 and T4 was found to be less in Vipos River compared with El Cadillal, indicating the decreased risk of the death of larvae and pupae in Vipos River compared with El Cadillal (Tables 7 and 8).

DISCUSSION

Most of the literature on anopheline mosquitoes has focused on the determination of the presence or absence of adult forms of malaria vectors in the different American countries (Rodríguez & Loyola 1989; Zimmerman 1992; Manguin et al. 1996; Dantur Juri et al. 2003, 2005, 2009, 2010a, 2010b). By focusing on the immature forms and their biology, Forattini (1962) provided detailed

Fig. 4. Cumulative risk for each transition (T1, T2, T3, and T4) over time (L: larvae, P: pupae, A: adult).

descriptions of aspects of anopheline breeding sites. Zimmerman (1992) later reported that most of the research into immature forms referred to the presence and/or absence of larvae and their habitat characteristics. Hoffmann & Samano (1938), Savage et al. (1990), and Fernández-Salas et al. (1994) associated the topography with the annual patterns of local rainfall in the formation of larval habitats of *An. pseudopunctipennis*. Thus, when the rainy season ends, larvae, floating plants, and green algae can be found in the larval habitats; the latter include pools, ponds, and lagoons that developed from the margins of rivers and streams.

Other studies of *An. pseudopunctipennis* were based on the larval habitats (Shannon 1930; Hoffmann 1931; Root & Andrews 1938; Aitken 1945; Levi-Castillo 1945; Rejmankova et al. 1991). In addition, Fernández-Salas et al. (1994) referenced the scarce studies in the ecological and biological aspects of the larvae of *Anopheles* mosquitoes. Therefore, the main objectives of this study were to determine the development time of the different stages, the relationship between the climatic seasons and local habitats on the time at which the specimens remain in each stage of development and on the transitions to the subsequent stages of metamorphosis or death in *Anopheles pseudopunctipennis* and *Anopheles argyritarsis*.

Anopheles pseudopunctipennis was collected not only in puddles resulting from the flooding of streams of El Cadillal Dam but also in Mountain Rivers of Rosario de la Frontera, Vipos, and Potrero de las Tablas. As was mentioned by Shannon & Davis (1927), Rozeboom (1941), Hackett (1945), Manguin et al. (1996), and Dantur Juri et
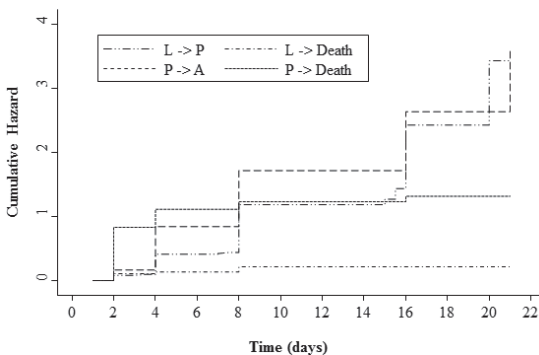
TABLE 4. PROBABILITY OF STAYING IN THE DIFFERENT STAGES (LARVAE, PUPAE, ADULT, AND DEATH) OVER TIME.

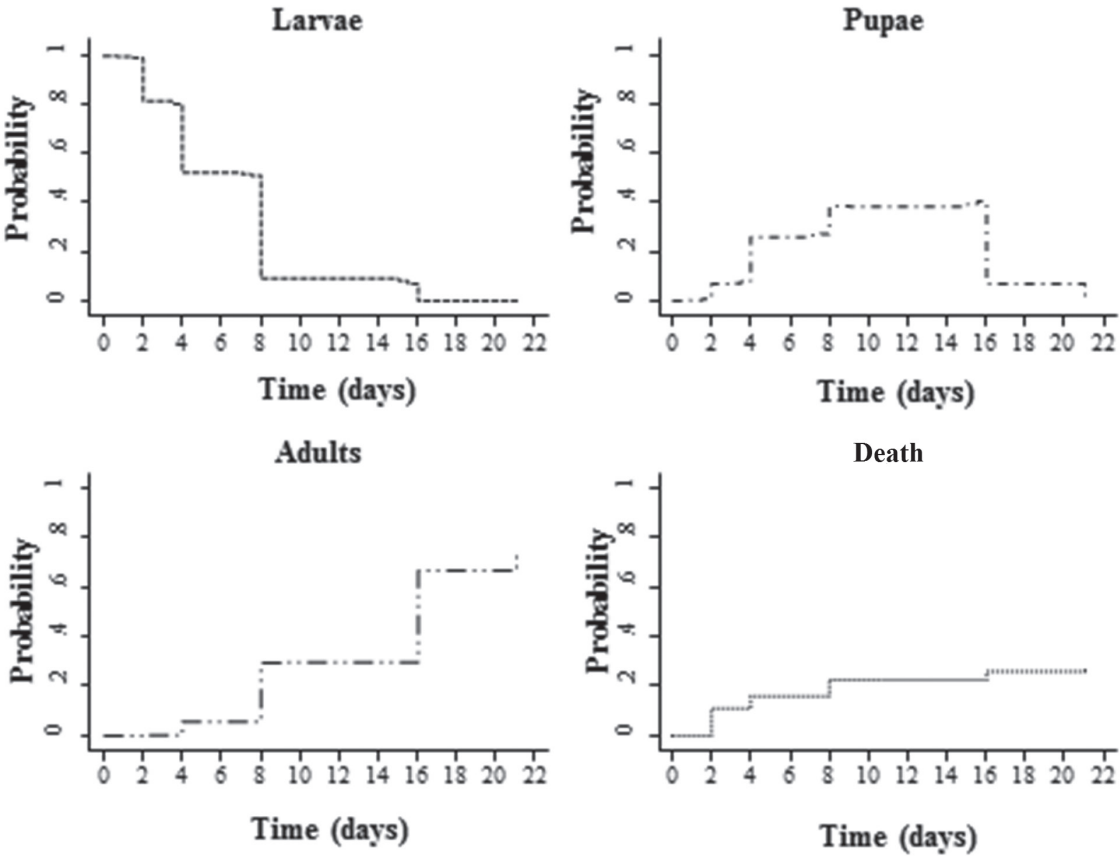| Time (days)	Larvae	Pupae	Adult	Death
0	1	0	0	0
1	0.99756	0.00244	0.00000	0.00000
1.5	0.99269	0.00731	0.00000	0.00000
2	0.81718	0.07130	0.00122	0.11030
3	0.81475	0.07374	0.00122	0.11030
3.5	0.80500	0.08349	0.00122	0.11030
4	0.52529	0.25899	0.05728	0.15844
7	0.51920	0.26508	0.05728	0.15844
7.5	0.51188	0.27239	0.05728	0.15844
8	0.09141	0.38574	0.29555	0.22730
9	0.09141	0.38452	0.29616	0.22791
15	0.08349	0.39183	0.29677	0.22791
15.5	0.06947	0.40585	0.29677	0.22791
16	0.00061	0.06886	0.66849	0.26204
20	0	0.06947	0.66849	0.26204
21	0	0.00000	0.73736	0.26264

Fig. 5. Probability of staying in one of the stages (larval, pupal, adult, and death) over time.

TABLE 5. EFFECT OF THE SPRING AND SUMMER SEASONS ON THE FOUR TRANSITIONS: LARVA TO PUPA (T1), LARVA TO DEATH (T2), PUPA TO ADULT (T3), AND PUPA TO DEATH (T4).

	Climatic seasons			
	Spring		Summer	
Transition (T)	Coefficient	*p-value*	Coefficient	*p-value*
1	0.0754	0.240	0.0350	0.740
2	-0.2266	0.140	**-1.4184**	**0.003**
3	0.0325	0.640	0.0731	0.500
4	-0.2045	0.290	**-0.8271**	**0.048**

al. (in press), *An. pseudopunctipennis* larvae appear more frequently in bodies of water with oxygen that are generally clean, exposed to the sun, and exhibit the presence of *Spirogyra* algae. In addition, throughout this investigation, the same characteristics were found in the larval habitats of *An. argyritarsis*.

Manguin et al. (1996) characterized the larval habitats of *An. pseudopunctipennis* in the countries of America and provided information related to the larval habitats, including other species of anopheline larvae. Thus, in addition to larvae of *An. pseudopunctipennis*, larvae of *An. aquasalis* (at sea level) and *An. argyritarsis* (in a wide range of distribution) were collected on Grenada Island.

In the present work, as was described by Mühlens et al. (1925) and Manguin et al. (1996), a strong association between the presence of *An. pseudopunctipennis* and the presence of *An. argyritarsis* was found because both species were found together, although as different proportions, in the same larval habitats.

We observed great seasonal fluctuation in the abundance of anopheline larvae with higher abundance during the autumn and spring seasons and a lower abundance during summer and complete absence during the winter. This finding is consistent with the results reported by Fernández-Salas et al. (1994). These researchers had found that *An. pseudopunctipennis* exhibited a marked seasonal fluctuation; because the larval habitats were negatively associated with the annual rainfall, and high abundance of larvae was found during the dry season.

In general, the studies conducted by Savage et al. (1990), Rejmankova et al. (1991), Berti et al. (1993), Fernández-Salas et al. (1994), Manguin et al. (1996), and Grillet (2000) focused on ecological aspects of anopheline larvae. In particular, Savage et al. (1990) and Rejmankova et al. (1991) reported on ecological aspects of *An. pseudopunctipennis* and *An. albimanus* larvae and the environmental factors that favor their presence; and asserted that the amount of filamentous algae and altitude as determinants of larval abundance. In addition, Fernández-Salas et al. (1994) referred to the possibility of finding larvae of *An. pseudopunctipennis* in different environments, such as plantations, forests, villages, cities, and swamps. Moreover, although most larval habitats are associated with streams and pools of stream banks, larvae are also found in ditches, ponds, lagoons, and holes in rocks. In addition, larvae have also been found in artificial containers, such as tanks, fountains (Rozeboom 1941), paddy fields, and marshy meadows (Downs et al. 1948). In the present study, *An. pseudopunctipennis* was the most abundant species in the puddles of El Cadillal Dam, and *An. argyritarsis* was the most abundant species in Potrero de las Tablas

TABLE 6. EFFECTS OF THREE LOCAL HABITATS ON THE FOUR TRANSITIONS: LARVA TO PUPA (T1), LARVA TO DEATH (T2), PUPA TO ADULT (T3), AND PUPA TO DEATH (T4).

	Localities					
	Potrero de las Tablas River		Rosario de la Frontera River		Vipos River	
Transition (T)	Coefficient	*p-value*	Coefficient	*p-value*	Coefficient	*p-value*
1	0.1949	0.011	0.2256	0.004	-0.2260	0.028
2	0.3771	0.042	0.3814	0.050	0.8221	0.000
3	0.0229	0.780	-0.0260	0.770	-0.0910	0.420
4	-0.2520	0.300	0.1018	0.670	0.6043	0.020

Rosario de la Frontera River (S 25° 48' W 64° 58', 791 m asl) is located in Salta Province. Potrero de las Tablas River (S 26° 21' W 65° 21', 950 m asl) and Vipos River habitat (S 26°29' W 65° 21', 786 m asl) are situated in Tucumán Province.

TABLE 7. α PARAMETER AND ITS EFFECT ON THE CLIMATIC SEASONS AND THE LOCALITIES.

Factors	Alpha (a)
Spring season	-0.28461176
Summer season	**-0.41932767**
PT Locality	-0.18419720
RF Locality	-0.05354408
V Locality	**0.84045516**

PT, Potrero de las Tablas River in Tucumán province; RF, Rosario de la Frontera River in Salta province.

The α parameter measures the overall effect of each covariate on the risk of each of the transitions.

Mountain River. Hence, it could be stated that is the current findings are consistent with those previously reported.

In this work, the survival of the different stages was analyzed through multistate models. The number of individuals that made the transition from larva to pupa was higher than the number that made the transition from pupa to adult. We supposed that even though the larval stage was much more subjected to external agents that can affect development, it could survive most challenges. This suggests that the pupal stage is the more delicate and vulnerable.

In addition, the probability of being in the larval stage is close to 100% until the second day after hatching at which point it starts to decrease. This information is vitally important for control programs focused on the breeding sites of the immature stages. Managers of control programs need to know the precise moment at which to attack the larval stage. The results of this study show that the fraction of the population declines after the second day of the development of the first stage larvae. Thus the timing of larvicides must be made early to achieve a great reduction in the larval population, which reduction will be reflected in the subsequent pupal and adults populations. Indirectly, effective reduction of the larval population can influence the potential transmission of malaria.

With respect to the co-variables, which represent the climatic seasons and the local habitats, the transitions from larvae to death and from pupae to death were found to be significant in the summer when the risk of larval death decreased, and the same was observed with the pupae.

The significant transitions of the larvae and pupae during the summer plus decreased mortality during this season demonstrate the important effect of this climatic season on the presence of the larvae that will become pupae and adults. Generally, it is reported that the major abundance of larvae of *Anopheles* occurs during the spring or autumn seasons, when the rainfall has decreased. During the summer, heavy rains increase river flow, which eliminates breeding sites of the immatures (Dantur Juri et al. in press). Also, Fernández Salas et al. (1994) reported that in Mexico the seasonal rainfall was directly and negatively associated with larval abundance of *An. pseudopunctipennis*. Heavy rainfall generally causes a decrease in the number of immature mosquitoes in both permanent and transitory habitats.

But Dantur Juri et al. (in press) also reported that in several breeding sites called foothill ravines of Aguas Blancas, which are close to Bermejo (Bolivia), the major abundance of *An. pseudopunctipennis* occurred during the spring and summer, i.e., similar to high abundance in breeding sites in this study. Based on this information, efforts should be made to exert a larval control in the breeding sites to avoid the major seasonal abundance of the larvae and pupae and indirectly of the adult malaria vectors.

The analysis of the effect of the local habitats on the transitions revealed that Rosario de la Frontera, Potrero de las Tablas and Vipos Rivers have a significant effect on the transition from larvae to death. Knowledge of the localities where the transition from larvae to death is significant should be a key consideration in deciding when to apply control measures at various breeding sites. These rivers are situated along a national road, where there is an intensive traffic of people throughout the year, and especially during the summer season. Measures to eliminate the larvae forms from these rivers could be an important step to prevent the production of adult vectors that attack travelers in this region.

As was shown in the present investigation, information on the biological and ecological aspects, including the seasonal fluctuations, of the larvae of the known malarial vector *An. pseudopunctipennis* larvae, as well as *An. argyritarsis*, which likely plays a role in the transmission of malaria, will raise awareness of the greater abundance of adult mosquitoes during the epidemiologically important spring season. These results will also raise awareness of the sur-

TABLE 8. γ PARAMETER AND ITS EFFECTS ON THE TRANSITIONS (L: LARVAE, P: PUPAE, A: ADULT).

Transitions	L -> P (T1)	L -> Death (T2)	P -> A (T3)	P -> Death (T4)
Gamma (γ)	-0.5685548	**0.7109526**	-0.2737883	**0.8543723**

γ represents the intensity of the effect of each covariate on each transition.

vival time of larvae (48 h) and the relationship between the climatic seasons and localities on the time at which the specimens remain at each stage and the transitions to different stages, i.e., summer was observed to be the season that exerts the most influence on the transitions from larvae to death and from pupae to death, and Vipos River was the locality in which both of these transitions was lower, resulting in a reduced risk of larval death in this locality. These results are very important when considering different strategies for the malaria control through the mosquito control in the larval habitats.

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