PACIFIC JOURNAL OF MEDICAL SCIENCES
{Formerly: Medical Sciences Bulletin}
ISSN: 2072 – 1625

Pac. J. Med. Sci. (PJMS)
www.pacjmedsci.com. Email: pacjmedsci@gmail.com.

CLUSTER ANALYSIS OF ANOPHELES STEPHENSI FOURTH INSTAR LARVAE BEHAVIOR TO EXPLORE SEQUENTIAL ORGANISATION OF LARVAE MOVEMENT IN A MICROCOSM.

***Rodney L Itaki[1], Setsuo Suguri[2], Arif-Ul-Hasan[2], Chigusa Fujimoto[3], and Masakazu Harada[2]**

[1]Immanuel Lutheran Rural Hospital, P.O Box 420, Wabag, Enga Province; itaki7@gmail.com
[2]Department of International Medical Zoology, Faculty of Medicine, Kagawa University, Miki, Kita, Kagawa, 761-0793 Japan; Suguri@med.kagawa-u.ac.jp; ahasan@med.kagawa-u.ac.jp; mharada@kms.ac.jp
[3]Department of Medical Technology, Faculty of Health Sciences, Kagawa Prefectural College of Health Sciences, Hara, Mure, Takamatsu, Kagawa 761-0123, Japan fujimoto@chs.pref.kagawa.jp
(*Corresponding author: Rodney L Itaki: itaki7@gmail.com)

CLUSTER ANALYSIS OF ANOPHELES STEPHENSI FOURTH INSTAR LARVAE BEHAVIOR TO EXPLORE SEQUENTIAL ORGANISATION OF LARVAE MOVEMENT IN A MICROCOSM.

***Rodney L Itaki[1], Setsuo Suguri[2], Arif-Ul-Hasan[2], Chigusa Fujimoto[3], and Masakazu Harada[2]**

[1]Immanuel Lutheran Rural Hospital, P.O Box 420, Wabag, Enga Province; itaki7@gmail.com
[2]Department of International Medical Zoology, Faculty of Medicine, Kagawa University, Miki, Kita, Kagawa, 761-0793 Japan; Suguri@med.kagawa-u.ac.jp; ahasan@med.kagawa-u.ac.jp; mharada@kms.ac.jp
[3]Department of Medical Technology, Faculty of Health Sciences, Kagawa Prefectural College of Health Sciences, Hara, Mure, Takamatsu, Kagawa 761-0123, Japan fujimoto@chs.pref.kagawa.jp
(*Corresponding author: Rodney L Itaki: itaki7@gmail.com)

ABSTRACT:

Experiments were conducted on 20 fourth instar Anopheles stephensi larvae to explore behavior organisation. Twenty fourth instar larvae were placed in a glass aquarium and filmed using a handy cam video recorder and the recordings analysed in a laptop computer. Data of transitions from one behavior to another for all observations were collated into a matrix of preceding and succeeding behaviors to study sequential organisation and relationship among behaviors. Significant testing for first-order transition was done using G test at $P< 0.005$ and a kinematic graph constructed from significant transitions. A time budget and transition frequency data constructed for each behavior were subjected to cluster analysis to explore relationship between the behaviors.

Result of the analysis showed that fourth instar Anopheles stephensi larvae behaviors occur in clusters in specific locations in their aquatic environment. Furthermore, the sequential organisation of behaviors is influenced by behavior frequency and the amount time a larva spent doing that behavior. When food is kept constant, other factors such as gas exchange requirements, behavior variation due to day-night cycle, presence of a predator, interlarval competition for food and the size of the aquarium (depth and width) maybe determining behavior organization.

Keywords: Anopheles stephensi, behavior organisation, cluster analysis
Received: January 2011: Accepted: April 2011

INTRODUCTION:

Global efforts to control malaria have been impeded by insecticide resistant vectors, drug resistant parasites and socioeconomic obstacles [1]. In an effort to search for novel control strategies larval behavior have been studied extensively [2, 3]. Certain mosquito species that were previously regarded as non-vectors have become a threat to humans as they find new habitats to establish themselves and the emergence of these potential vectors has been attributed to the changes in the environment and the influence of modern life [4, 5]. Anopheles stephensi has been observed to quickly establish itself in a new environment [6]. The ability of a mosquito species to establish itself in a new environment therefore is directly affected by the ability of the larva to exploit its aquatic habitat [6] and there is a need to re-examine larva behavior in its aquatic environment that may shed some light on factors determining a species' ability to establish itself in a new environment.

Differences in larval behavior, especially in relation to feeding, may result in different abilities to exploit their habitat [7]. Species-specific differences in larval behavior [3, 8] may also allow certain species to adapt more quickly to new environment and establish themselves rapidly [7]. Walker and Merritt [2] created a catalogue of larval behaviors using Aedes triseriatus in an environmental with a constant food supply and noted that Aedes triseriatus used their entire habitat for feeding. Yee et al [3] compared the feeding behavior of Culex pipiens, Aedes albopictus and Ochlerotatus triseriatus (formerly Aedes triseriatus), all container-breeding mosquitoes, in two different food environments and found significant differences in larval behavior among the species.

Anopheles species generally feed at the air-water interface but they can also dive and feed at the bottom of their aquatic habitats [8, 9, 10, 11]. This diving ability in Anopheles species is most evident as an alarm response [9, 11]. Inter-specific competition between sibling species of Anopheles arabiensis and Anopheles gambiae has been reported by Schneider et al. [12].

Anopheles stephensi is a recognized malaria vector in Asia [6]. Using this species as a model, we conducted experiments to determine the behavior organisation of fourth instar Anopheles stephensi larvae in a microcosm to explore patterns of behavior that can provide insights into understanding this species' ability to establish itself in a new environment.

MATERIALS AND METHODS:

Twenty fourth instar Anopheles stephensi were used in the experiments. Mosquito larvae and adults were maintained using standard

protocols in an insectary with temperature maintained at 26°C and relative humidity 65 % with a 15 hours 8 hours day night cycle [11]. Light was provided by four 40-watt fluorescent light bulbs. Eggs were hatched in 250ml of de-chlorinated tap water in plastic cups (surface area = 95 cm^2). At the late second to early third instar stage, larvae were transferred to 33- x 24- x 7-cm pans. Larvae were fed on ®Tetra Min baby fish food. Water was changed every other day.

The observation experiments were done in a 59- (length) x 28- (depth) x 35-(height) cm glass aquarium. The aquarium was filled with de-chlorinated tap water and incubated for one week to permit growth of microorganisms on the walls including the floor, in the water column and at the air-water interface [2]. A few pebbles were also put in the aquarium to allow microorganisms to grow on their surface. Twenty fourth instar larvae were individually pipetted into the aquarium and allowed to acclimate for one hour before larval behaviors were recorded. Observation data was collected from the 20 larvae.

Walker and Merritt [2] and Clements [10] have catalogued the list of behaviors observed in larval behavior studies. We used the same definitions with minor modifications to suit our experiment design. Some behaviors that were observed by Walker and Merritt [2] were not included in our study and others were combined. Briefly, the definitions of behaviors used in our study were as follows:

- Float/suspension feed – the larva is attached to the water's surface via its respiratory siphon with the body hanging obliquely into the water column. Anopheles larva lies horizontally in line with the air-water interface. The larva may be still or move slowly as a result of brush movements.
- Float/interfacial feed – the larva is attached to water's surface and its body bent into a U shape so that its mouthbrushes makes contact with the air-water interface. Anopheles larva attaches itself in parallel with the water's surface and rotates its head 180 degrees to make contact with the air-water interface.
- Autogroom – At either the surface or underwater, a larva bends its body into a U shape and works its mouthparts against its own body.
- Dive – A larva spontaneously descends from a position near the water surface using a wriggling, swimming motion.
- Brushwall – A larva that is underwater and its siphon detached from the air-water interface brushes the wall of the observation chamber with its mouthparts. The larva may be still or moving.

- Wriggleswim – A larva moves through the water column by flexing and unflexing movements of its entire body forming a wriggling motion.
- Underwater/mouth swim – A larva moves forward in the water column as a result of its suspension feeding movements, not by flexing its body. The larva is not attached to the water's surface.
- Allogroom/feed – A larva directs its mouthparts against a nearby larva.
- Underwater/still – A larva remains motionless while underwater, usually at the bottom of the water column.
- Rise – A larva, when underwater, ascends through the water column to the surface.
- Float/brushwall – the larva is positioned at the surface while attached to the air-water interface via its siphon and brushes the wall of the observation chamber with its mouth parts. The larva may be still or moving.
- Bottom feed – A larva after diving and reaching the bottom of the water column brushes the floor, pebbles or chews a substrate. A larvae brushing the wall of the observation chamber approximately 1-2cm from the floor was also regarded as bottom feed. This behavior combines the float/substrate brush and chew substrate behaviors that were observed by Walker and Merritt [2].

A larva in the aquarium in any behavioral state was chosen at random and filmed for five minutes using a handy-cam (Sony Co. Japan) video recorder. To enhance image contrast, recordings were done with a white card placed behind the aquarium in daylight conditions [13]. Twenty fourth instar larvae were placed in the observation aquarium and allowed to acclimatize for one hour prior to filming. A larva was than chosen at random and filmed for five minutes. After videotaping 10 larvae, all 20 larvae in the aquarium were removed and replaced with a new group of 20 fourth instar larvae. This was done to ensure a larva was not filmed twice. Again one hour of acclimatization time was allowed and 10 larvae were videotaped at random, each larva being recorded for five minutes.

The focal-individual sampling method was possible because the tempo of larval behavior and the low density of larvae in the aquarium allowed the observer to track an individual larva [2] and videotape its behavior. Great care was taken not to videotape a larva more than once. The observer became familiar with larval behaviors through preliminary observation of more than 300 fourth instar larvae over a 10 month period.

Video tape recordings were converted to DVD and viewed on a Mac Os X version 9.3 laptop

computer. To study sequential organization and relationship among behaviors, data of transitions from one behavior to another for all observations were collated into a matrix of preceding and succeeding behaviors with the diagonal of the matrix held at logical zero, assuming that a behavior can not follow itself.
The transition matrix was collapsed about the cell of interest to form a 2x2 contingency table [2] and first-order transitions for significantly greater occurrence than expected by chance was tested using G test at P <0.005 [14, 15]. Statistical significance testing was done using Excel set up on a website [15]. To visualize the organization of the behaviors in the microcosm, a kinematic graph of the behavioral sequences were constructed from the transition matrix by showing frequencies of significant nonrandom transitions between behavior states [2, 16].

Data from the time budget and transition matrix were subjected to hierarchical cluster analysis to explore relationships between the different larval behaviors. Cluster analysis was done using TANAGRA [17].

RESULTS AND DISCUSSION:

Behaviors were recorded from a total of 20 fourth instar Anopheles stephensi larvae. Each larva was recorded for five minutes totaling 110 minutes of observation time (Table 2). Anopheles stephensi larvae spent majority of their time in the float/interfacial feed and bottom feed states (Table 2).

The 12x12-transition matrix (Table 1) shows the frequencies of transition among behavioral states. Zero entry indicates no transition was observed between these behaviors. Entries with an asterix indicates a significantly greater frequency of transition from the preceding to the succeeding behavior than expected by chance (G test, P<0.005).

The kinematic graph (Figure 1) shows statistically significant (G test P<0.005) patterns of association between behaviors based upon the frequency of occurrence of transitions between the paired behaviors. Patterns of association that were not statistically significant are not shown. The mean time spent in the behavioral state is shown in brackets. The numbers along the arrows represent percentage of transitions from preceding to succeeding behavior. Generally, the kinematic graphs show that there were a group of behaviors that occurred near the water's surface and another group of behaviors occurred underwater.

These two groups were connected by dive and rise behaviors. Wriggleswim was used to transit between one surface behavior with another. Larvae also used wriggleswim to move from one underwater behavioral state to another. These three behaviors dive rise and wriggle-

swim therefore can be called transition behaviors, as larvae used these behaviors to transit between one behavioral state and another.

The dominant activity at the surface was float/suspension feed and float/interfacial feed with frequent transitions occurring between these two behaviors. There were also frequent transitions between float/suspension feed and float/brushwall. This was because larvae that were in float/suspension feed state would frequently move along the water's surface and bump into the wall of the observation chamber and brush the wall. There were also frequent transitions between allogroom/feed and both float/suspension feed and float/interfacial feed. It was also interesting to note that Anopheles stephensi larvae immediately moved away from each other upon contact.

Transition from float/suspension to dive was not a significant behavior. Comparison of diving behavior between aedes, culex and anopheles species have shown that diving is not a frequent behavior in anopheles species [10, 11]. However, the few times that a larva did break from the water's surface; it would usually swim right to the bottom as reflected by significant transitions from dive to bottom feed (Figure 1). Once a larva dived to the bottom of the aquarium it spent a long time feeding before surfacing. The diving pattern of Anopheles stephensi fourth instar larvae was in a zigzag manner with periods of sinking passively.

Cluster analysis of the frequencies of behaviors and the mean time allowed a different interpretation of the data. Cluster analysis of the time budget data showed the behaviors were to be clustered into five groups (Figure 2) while the frequency data clustered into seven groups (Figure 3). The dendrogram showing results of hierarchical cluster analysis on mean time (Figure 2) showed autogroom, allogroom/feed, brushwall and float/brushwall were closely related and formed one cluster that was closely linked with underwater/mouthswim. These five behaviors were loosely linked to the cluster formed by wriggle-swim and float/suspension.

Rise and underwater/still formed one cluster that was distantly linked to the cluster formed by float/interfacial feed and dive. These four behaviors were also loosely linked to floor feed. Hierarchical cluster analysis on data from the frequency transition matrix (Figure 3) showed dive and rise formed one cluster that was loosely linked to the cluster formed by floor feed and wriggleswim.

Autogroom/feed and underwater/mouthswim formed a cluster that was closely related to underwater/still. These three behaviors were loosely linked to the cluster formed by float/brushwall and brushwall. Allogroom/feed and float/interfacial feed formed a cluster that

was loosely linked to the rest of the behaviors.

The larval behaviors observed in this study can be generally classified into three groups: (i) those that were performed near the water's surface, (ii) those that were performed at the bottom of the observation chamber and (iii) behaviors that can be termed as transition behaviors. Walker & Merritt [2] when cataloguing larval behaviors also classified the behaviors into a group that occurred near the surface and a group that occurred underwater and these two groups of behavior were connected by dive and rise behaviors. We have grouped dive, rise and wriggleswim into what we have termed as transition behaviors. Transition behaviors connected the near surface behaviors with the bottom behaviors, two surface behaviors or two bottom behaviors. Cluster analysis of the behavior frequency data revealed dive and rise formed a cluster supporting our hypothesis but wriggleswim formed a cluster with bottom feed. This is because wriggleswim was a frequent behavior performed while feeding at the bottom of the aquarium. Cluster analysis of the time budget data showed a different pattern. The transition behaviors were clustered with other behaviors, in particular float/suspension feed and float/interfacial feed.

The kinematic graph (Figure 1) obtained from significant preceding-succeeding transition showed patterns of behavior observed near the water's surface and at the bottom of the aquarium. Similar observations were obtained when the same data were subjected to cluster analysis (Figure 3).

Allogroom/feed, float/interfacial feed, float/brushwall, brushwall and float/suspension feed were all behaviors observed near the water's surface and which were closely linked as shown by cluster anlaysis (Figure 3). The bottom behaviors - underwater/still and bottom feed formed also formed a cluster of their own (Figure 3).

Cluster analysis of the time budget data although revealed five clusters of behavior, was able to separate the bottom behaviors from the behaviors observed near the water's surface (Figure 2). Autogroom, allogroom/feed, brushwall and float/brushwall were behaviors observed near the water's surface and formed one cluster (Figure 2). Bottom feed and underwater/still also formed their own clusters in association with one of the transition behaviors (Figure 2).

We conducted our study without varying the food environment therefore we might have missed some larval behaviors such as patterns of intra-specific competition, use of air-bubbles to breath underwater, different modes of feeding and different modes of swimming that have been described in previous studies [2, 3, 12, 13].

Our recording time of five minutes may have also limited us from videotaping other previously described larval behaviors. However, by grouping different specific behaviors into one general group with a common theme (e.g. bottom feed for different mode of feeding behaviors observed at bottom of aquarium) we tried to overcome this limitation.

The larval behaviors observed and described are for fourth instar larvae only. These behaviors may not be the same for other stages of larvae. We choose the fourth instar stage because they are larger and easier to observe and videotape their movements. Furthermore, the behaviors were recorded in day light conditions. This might have some influence on larval movement. We do not know if there will be any difference in larval movement in night conditions which has the potential to affect the overall behavior organization.

Microcosm scaling has been shown to affect experimental results when studying aquatic insects [18] and may have also influence the results of our study.

CONCLUSION:

This study showed that cluster analysis of Anopheles stephensi 4th instar larva behavior observation data when used in combination with a pictorial representation of significant sequential organisation of behavior can reveal patterns of behavior that has the possibility of being exploited to develop new larval control methods or improve existing ones.

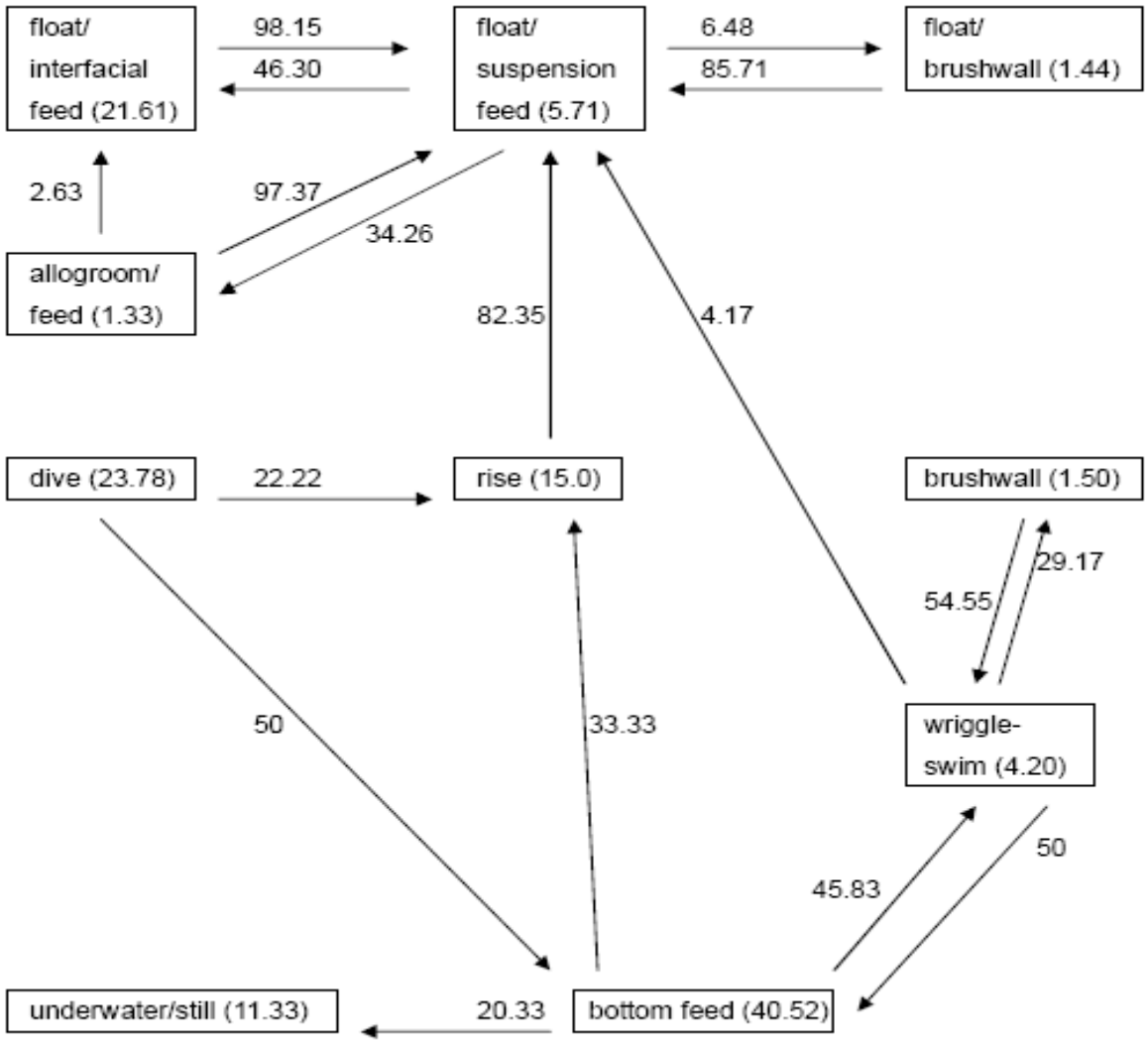
However, further studies are needed to determine what the patterns of behaviors observed mean biologically.

ACKNOWLEDGMENT:

This work was supported in part by a Grant-in-Aid for Scientific Research [KAKENHI (174060080)] from the Ministry of Education, Culture, Sports, Science and Technology. We would also like to thank the Government of Japan for financially supporting Dr. Rodney L. Itaki and Dr. Hasan Arif through its Monbusho scholarship program.

Figure 1: Kinematic graph of Anopheles Stephensi 4th instar larvae.

{Kinematic graph showing statistically significant transitions (G test P<0.005) and mean time spent (seconds) performing each behavior. The percentage of total transitions from the preceding to the succeeding behavior is next to each arrow. The number in brackets next to the behavior represents the mean time}.



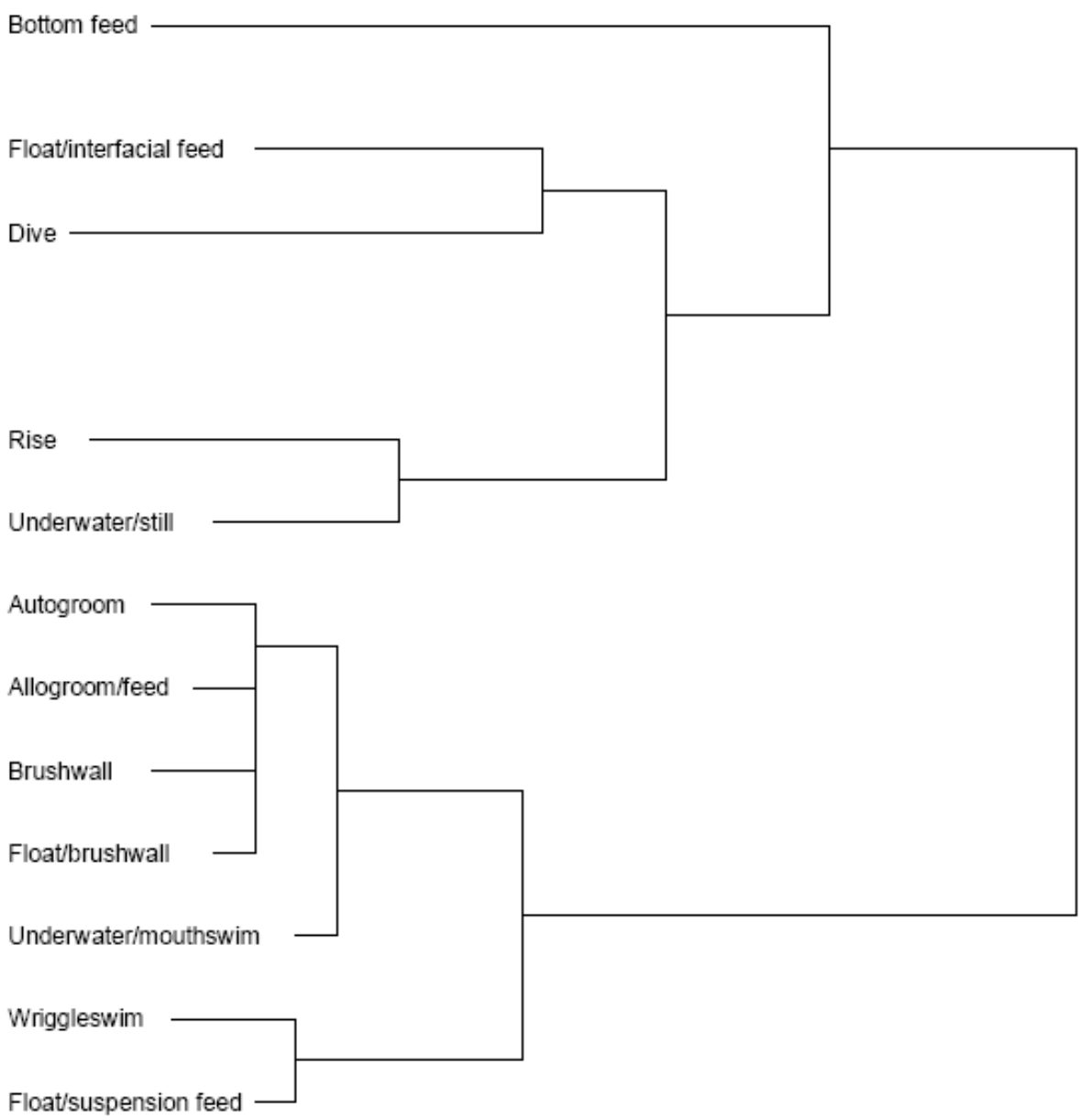


Figure 2: Dendrogram showing results of hierarchical cluster analysis of data from time budget for Anopheles stepensi 4th instar larvae.

{Dendrogram is not drawn to scale. Closely allied behaviors are indicated by relative length of the branches. Cluster analysis done using TANAGRA [17]}.

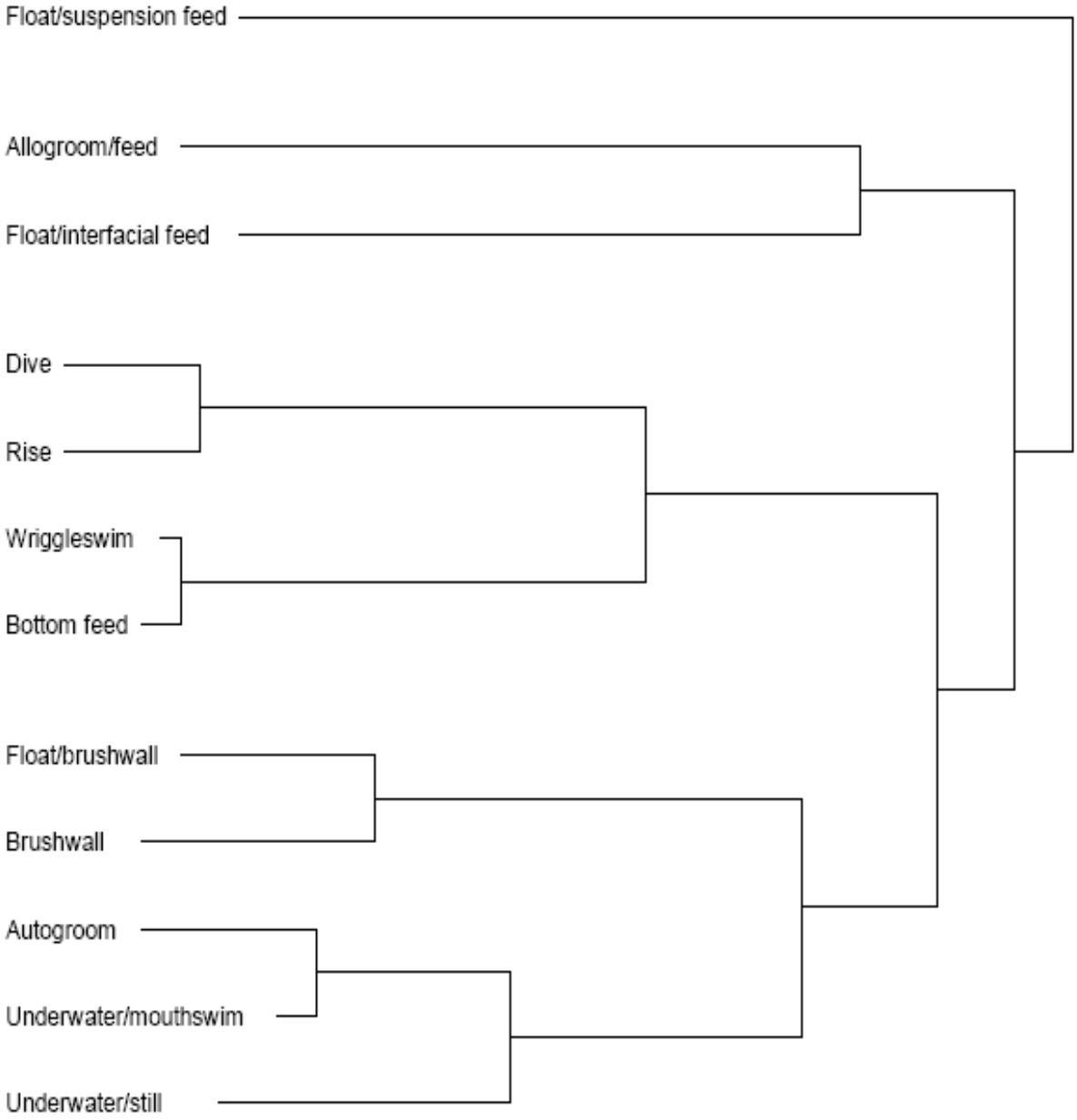


Figure 3: Dendrogram showing results of hierarchical cluster analysis of data from transition matrix for Anopheles stephensi 4th instar larvae.

{Dendrogram is not drawn to scale. Closely allied behaviors are indicated by relative length of the branches. Cluster analysis done using TANGARA [17]}

Table 1: Transition matrix for frequencies of transition for Anopheles stephensi 4th instar larvae; Entries with an asterix (*) indicate statistically significant transitions (G test, P<0.005).

Preceding behavior ↓ Succeeding behavior →

	Float-suspension feed	Dive	Wriggle-swim	Brush-wall	Bottom feed	Rise	Float/ brush-wall	Float/ inter-facial feed	Under-water/ still	Allo-groom/ feed	Under-water mouth-swim	Auto-groom
Float-suspension feed	-	11	0	0	0	0	7*	50*	0	37*	0	3
Dive	0	-	2	3	9*	4*	0	0	0	0	0	0
Wriggle-Swim	1*	1	-	7*	12*	1	0	0	2	0	0	0
Brush-wall	0	2	6*	-	0	0	0	0	0	0	0	0
Bottom feed	0	0	11*	0	-	8*	0	0	5*	0	0	0
Rise	14*	2	1	0	0	-	0	0	0	0	0	0
Float/ brush-wall	6*	1	0	0	0	0	-	0	0	0	0	0
Float/ interracial feed	53*	0	1	0	0	0	0	-	0	0	0	0
Under-water/still	0	0	0	0	6	0	0	0	-	0	0	0
Allogroom/ feed	37*	0	0	0	0	0	0	1*	0	-	0	0
Under-water mouth-swim	0	0	0	0	0	0	0	0	0	0	-	0
Autogroom	2	0	0	0	0	0	0	0	0	0	0	-

Table 2: Time budget for Anopheles stephensi 4th instar larvae behavior (n=20).

Behaviour	Duration (s) Mean ± SD	Range (s)	% Total time (Total time = 18600 sec)	Frequency
Float/suspension feed	5.71±5.03	1-81	11.08	56
Dive	23.78±9.54	3-36	10.68	11
Wriggleswim	4.2±7.79	1-33	2.9	20
Brushwall	10.50±8.73	3-29	2.9	8
Bottom feed	40.52±35.36	2-149	29.43	21
Rise	15.0±3.67	9-19	7.78	15
Float/brushwall	1.44±1.5	1-5	0.45	9
Float/interfacial feed	2.68±27.79	2-128	32.88	44
Underwater still	11.33±9.61	1-20	1.18	3
Underwater mouthswim	-	-	-	-
Allogroom/feed	1.33±0.87	1-4	0.55	12
Autogroom	1.67±0.58	1-2	0.17	3

REFERENCES:

1. Albuquerque MRJR, Silveira ER, Uchoa EDEA, Lemos TLG, Souza EB, Santigao GMP, Pessoa ODL. Chemical composition and larvicidal activity of the essential oils from Eupatorium betonicaeforme (D.C) Baker (Asteraceae). J Agri Food Chem 2004; 52: 6708-6711.
2. Walker ED, Merritt RW. Behavior of larval Aedes triseriatus (Diptera: Culicidae). J Med Entomol 1991;28(5):581-589.
3. Yee DA, Kesavaraju B, Juliano SA. Larval feeding behavior of three co-occurring species of container mosquitoes. J Vector Ecol 2004; 29(2):315-322.

4. Gould EA, Higgs S, Buckley A, Gritsun TS. Potential arbovirus emergence and implications for the United Kingdom. Emerg Infect Dis 2006;12(4):549-555.
5. Hayes EH, Komar N, Nasci RS, Montgomery SP, O'Leary DR, Cambell GL. Epidemiology and transmission dynamics of West Nile Virus disease. Emerg Infect Dis 2005;11(8):1167-1173.
6. Briet JTO, Galappaththy NLG, Amerasinghe PH, Konradsen F. Malaria in Sri Lanka: one year post-tsunami. Malar J 2006;5:42.
7. Duhrkopf RE, Benny H. Differences in the larval alarm reaction in populations of Aedes aegypti and Aedes albopictus. J Am Mosq Control Assoc 1990;6(3):411-414.
8. Workman PD, Walton WW. Larval behavior of four Culex (Diptera: Culicidae) associated with treatment wetlands in the southwestern USA. J Vector Ecol 2003;28(2):213-28.
9. Tuno N, Miki K, Minakawa N, Githeko A, Yan G, Takagi M. Diving ability of Anopheles gambiae (Diptera: Culicidae) larvae. J Med Entomol 2004;41(4):810-812.
10. Clements A N. Behavior and aspects of the biology of larvae and pupae. In: The Biology of mosquitoes. Volume 2. Sensory reception and behavior. CABI Publishing, 1999:134-205.
11. Itaki RL, Suguri S. Differences in submergence and alarm reaction rate in Anopheles stephensi, Aedes egypti and Culex pipiens fourth instar larvae in response to vibration stimulus. Pac J Med Sci 2010; 7(2): 44-49.
12. Schneider P, Takken W, McCall PJ. Interspecific competition between sibling species larvae of Anopheles arabiensis and An. gambiae. Med Vet Entomol 2000;14:165-170.
13. Brackenbury J. Locomotion through use of the mouth brushes in the larva of Culex pipiens (Diptera: Culicidae). Proc R Soc Lond 2001; 268:101-106.
14. Sokal RR, Rohlf FJ. Biometry: The Principles and Practice of Statistics in Biological Research. New York: W.H. Freeman New York, 1995.
15. Mcdonald JH. G-test of independence. 2006 [http://udel.edu/~mcdonald/statgtestind.html] (last viewed April 2006)
16. Sustare BD. Systems diagrams. In: Colgan PW, ed. Qualitative ethology. New York: Wiley, 1978:275-311.
17. Rakotomalala R. TANAGRA: a free software for research and academic purposes. 2005 [http://chirouble.univ-lyon2.fr/~ricco/tanagra/en/tanagra.html] (last viewed April 2006).
18. Wynn G, Paradise CJ. Effects of microcosm scaling and food resources on growth and survival of laval Culex pipiens. BMC Ecol 2001;1:3.