4. Simuliid biodiversity and environmental factors

Although 22 simuliid species were collected in total (detailed in 2.1) only 17 of these larval species are considered for detailed analysis here. Five species were excluded from detailed analysis because of difficulties with clear identification in the case of *Simulium asakoae* and *S*. sp. nr. *sheilae*, while *S. grossifilum* was not found in the larval stage and *S*. sp. D and *S*. sp. E were collected prior to the study period.

4.1 Sampling efficiency

The pooled sample shows a sampling intensity of ≈ 6 with an inventory completion index of ≈ 98. There was one singleton (*S. rudnicki*), two doubletons (*S. gombakense* and *S.* sp. B) and nine species represented by less than ten individuals. These data, and the species accumulation curve, with crossing singleton and doubleton curves, (Figure 89) shows that sampling has been quite effective and close to completion.

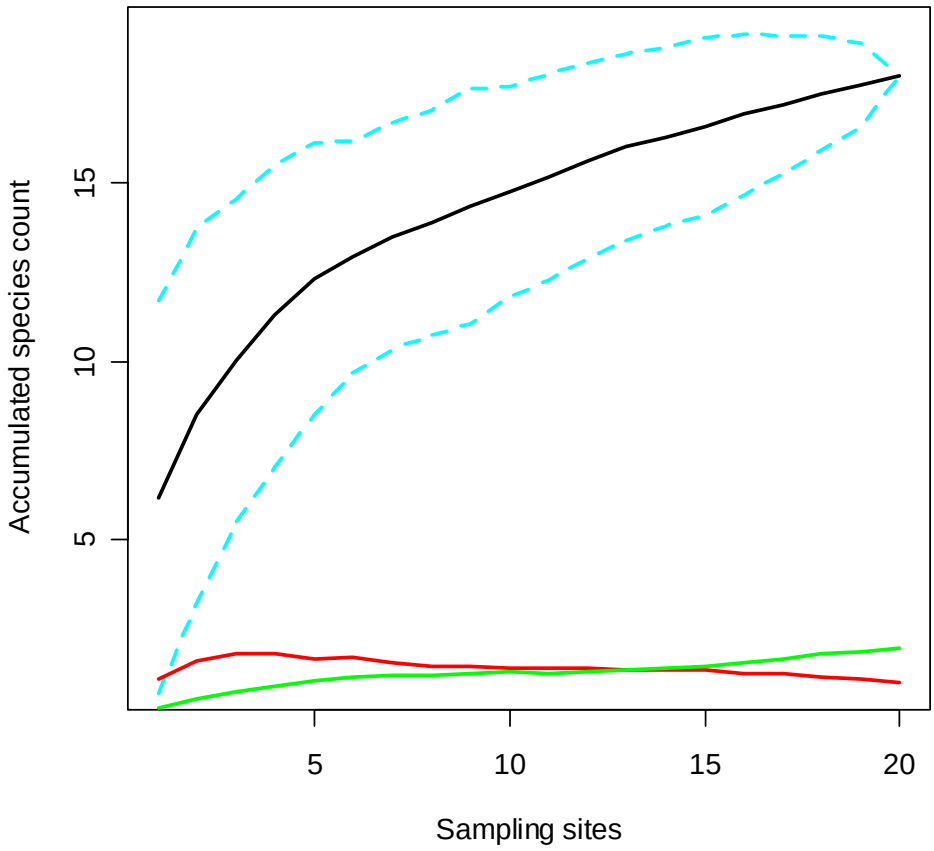

Figure 89 Species accumulation from 20 collection sites between July 2000 and June 2001 (solid line) with estimated 95% confidence limits (dashed blue) and singleton (red solid) and doubleton (green solid) curves

4.2 Environmental factors and biological associations

4.2.1 Abundance

Drought: Unsurprisingly this was the main factor affecting simuliid larval abundance. Simuliid larvae were totally absent from streams during drought periods. This resulted in a strong negative relationship between the period of drought a site experienced and abundance (ρ = -0.8052, $p < 0.001$; $r^2 = 0.5751$, $p < 0.001$; Figure 90).

The strong effect of drought on abundance means caution is required when examining other factors that may be affecting abundance.

Altitude: Larval abundance generally showed a positive correlation between total abundance and altitude (Figure 91). However, this relationship is mostly a result of the influence of drought, which also had a relationship with altitude i.e., lower altitude sites typically experiencing longer drought. Site 14 showed a marked departure from the altitude-abundance trend, as this site was a high altitude site that experienced drought. Site 4 was also unusual in having a high abundance as a result of a very high number of *S. nakhonense* at the start of the dry season (late September-December).

Water temperature: There was no association between median water temperature and abundance (Figure 92).

pH: Like water temperature there was no association between abundance and median water pH (Figure 93).

Stream size: Stream size, as indicated by maximum stream width, showed no strong relationship with abundance (Figure 94). Minimum stream width shows a similar pattern.

Canopy: Sites with an open canopy had significantly higher abundance than those with a closed canopy (Figure 95, $t = -2.8897$, $p < 0.05$).

Substrate: There were no significant differences in abundance among the different substrate types with the different substrates varying widely (Figure 96).

Human disturbance: Simuliid abundance was significantly higher in streams subject to human disturbance (Figure 97, $t = 4.2593$, $p < 0.01$).

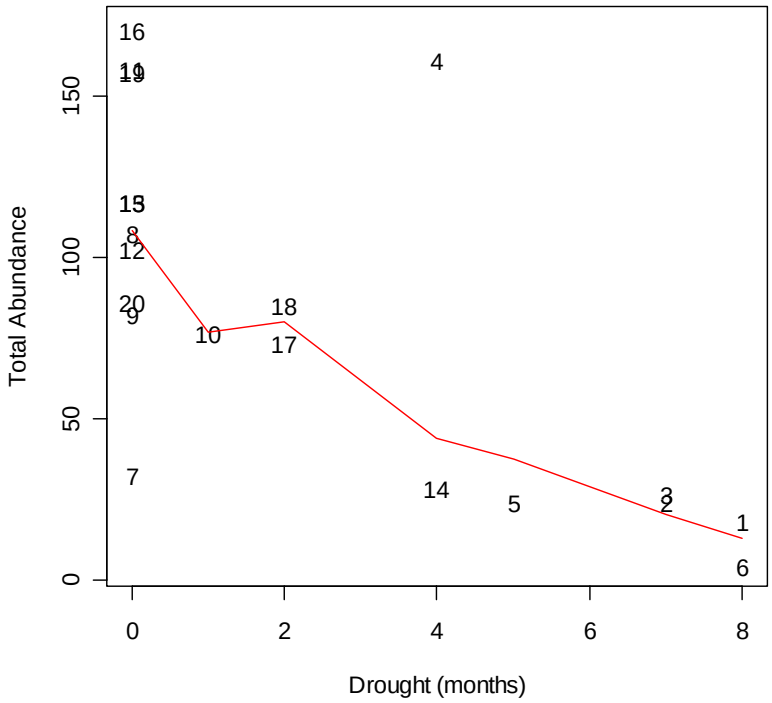

Figure 90 Drought and black fly abundance

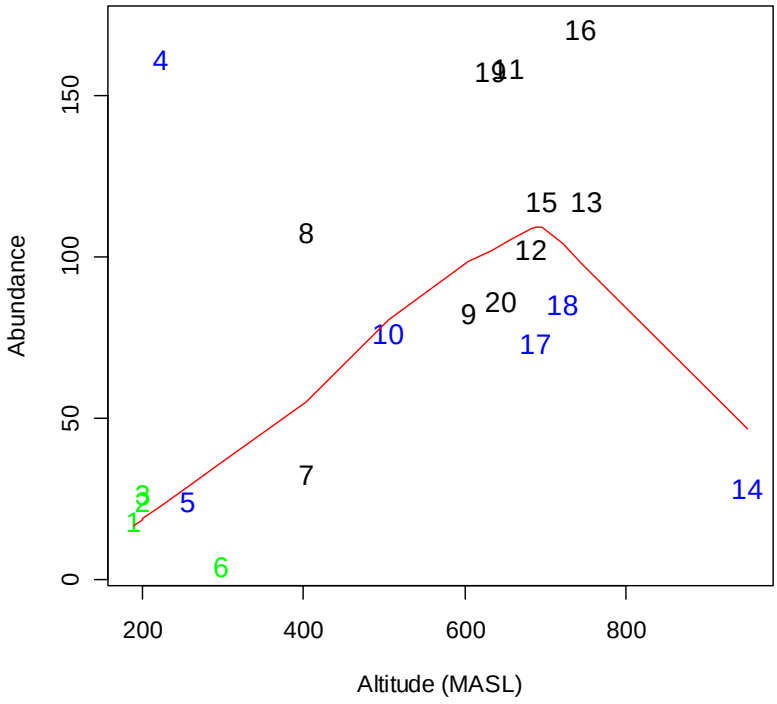

Figure 91 Altitude and black fly abundance. Black = no drought, blue = less than six months drought, green = six or more months drought

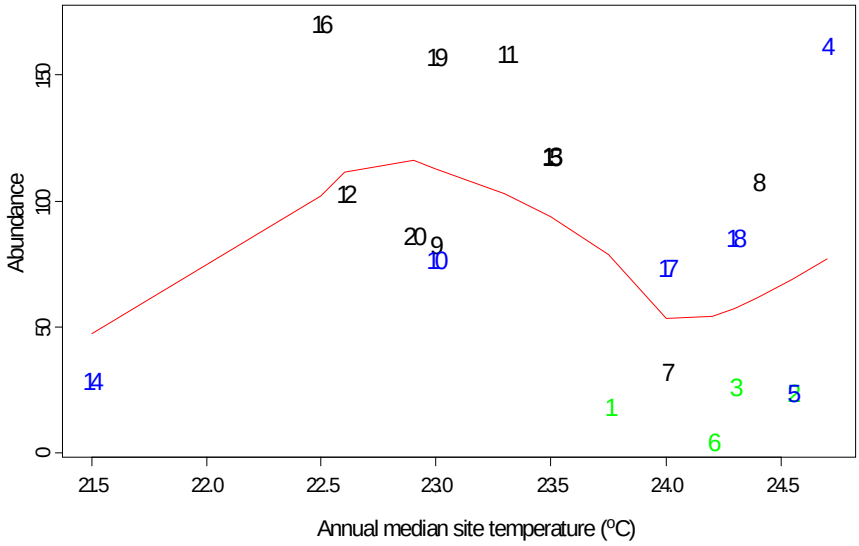

Figure 92 Water temperature and black fly abundance. Black = no drought, blue = less than six months drought, green = six or more months drought

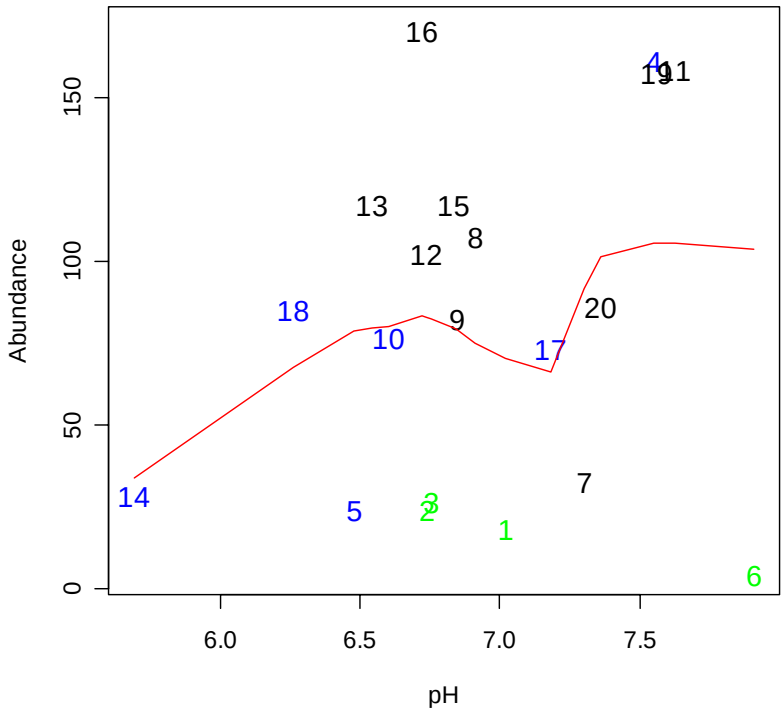

Figure 93 pH and black fly abundance. Black = no drought, blue = less than six months drought, green = six or more months drought

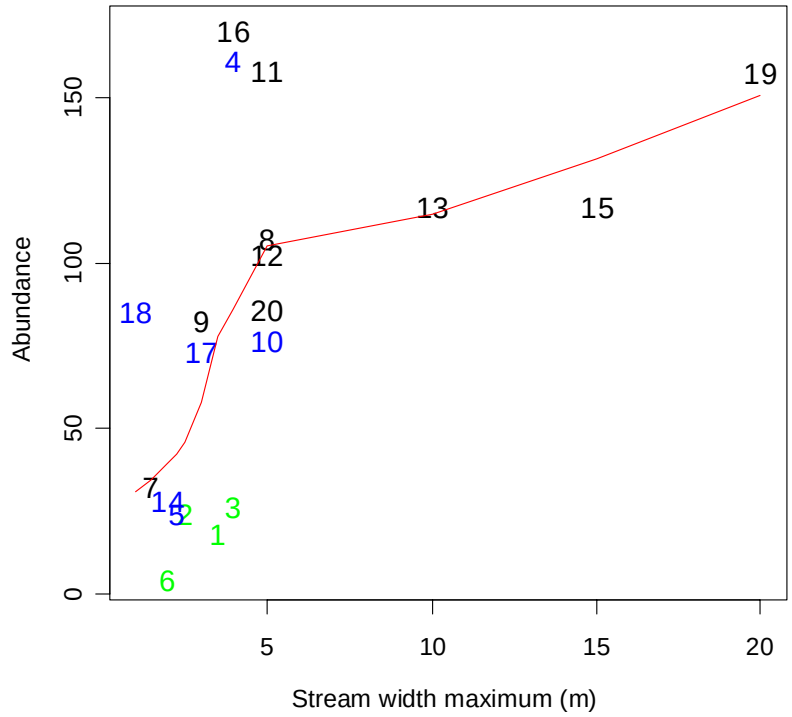

Figure 94 Stream size (max. width) and black fly abundance. Black = no drought, blue = less than six months drought, green = six or more months drought

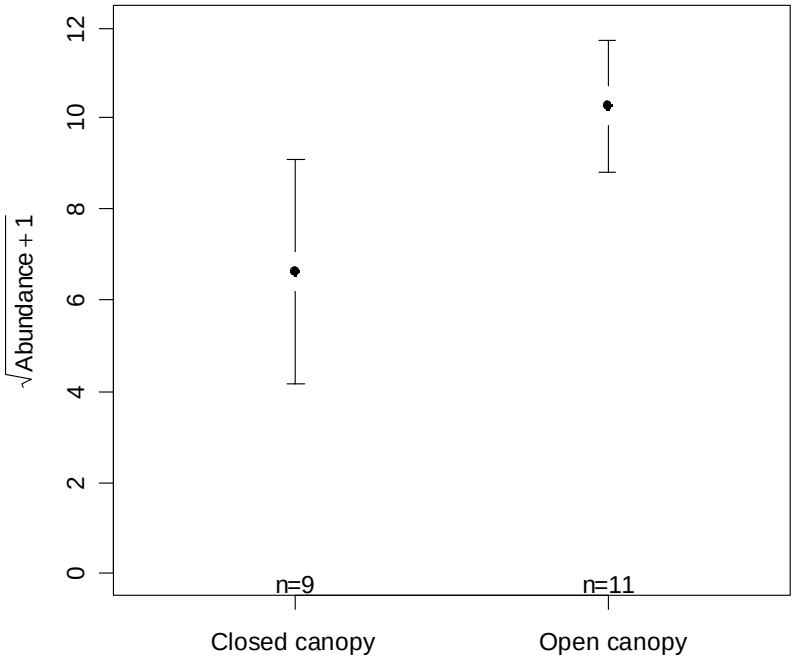

Figure 95 Canopy and black fly abundance

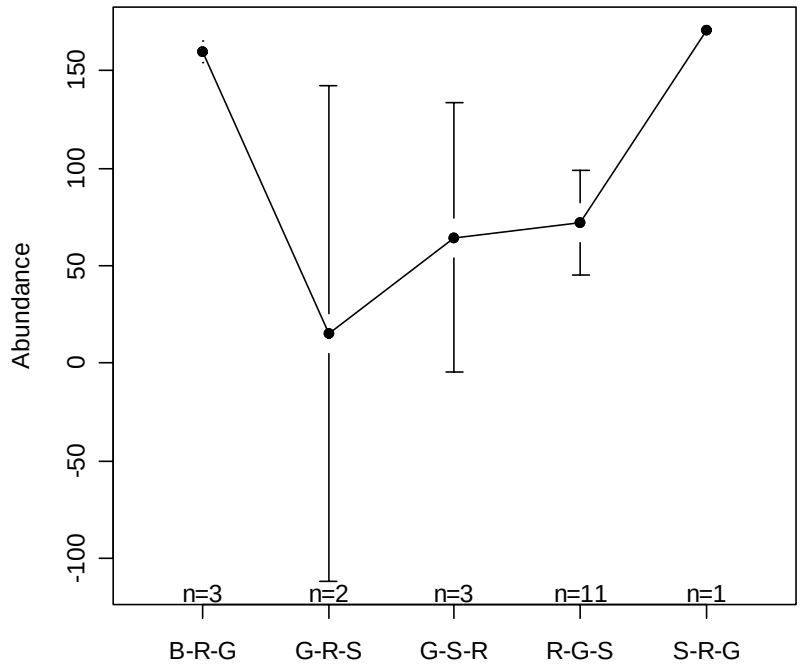

Figure 96 Substrate type and black fly mean abundance. B = bedrock, R = rock, G = gravel, and S = sand

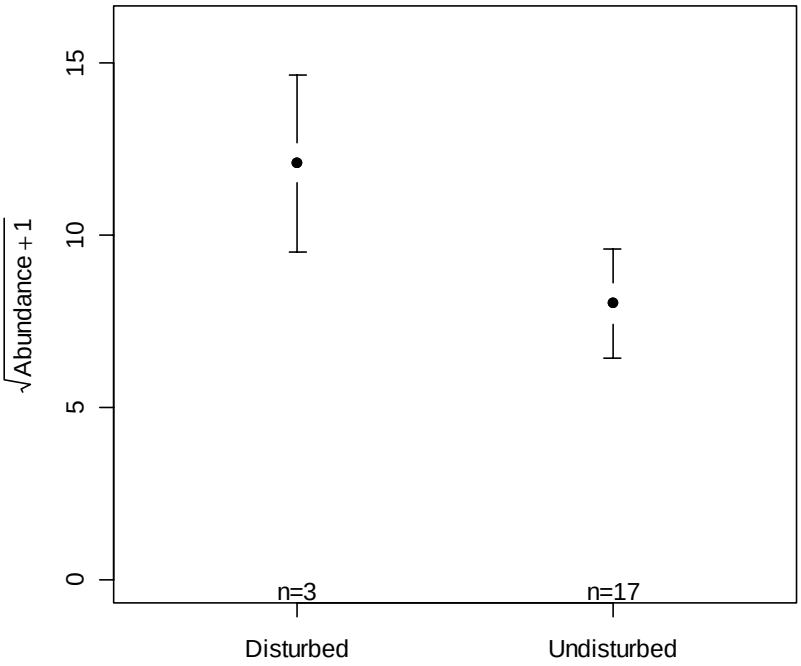

Figure 97 Human disturbance and black fly mean abundance

4.2.2 Species richness

Species richness was analyzed by examination of both species richness estimators as well as observed species richness.

4.2.2.1 Species richness estimates

The completeness of the sampling is again apparent when examining how close the observed species count of 17 species was to the various species estimators (18-24 species) (Table 7).

In addition to this overview of species richness estimates, the Chao 1 estimate of species was used to examine differences among sites and factors in detail.

Drought: The relationship between drought and the Chao 1 estimate of species richness is broadly similar to that seen between drought and abundance. There is generally a decreasing trend of estimated richness with increasing drought length (Figure 98; $\rho = -0.8159$, $p < 0.001$; $r^2 = 0.4093$, $p < 0.01$). However, there is a great deal of variation in the richness of sites not experiencing drought ranging from as low as 2 species at site 18 to 17 species at site 11.

Altitude: The relationship between altitude and the Chao 1 species estimate is similar to that seen for the altitude and abundance relationship (Figure 99) with a humped-relationship with middle altitudes sites having higher richness estimates that both higher and lower altitude sites. No doubt part of this relationship is a result of the mid-altitude sites being less drought prone. However, it is interesting that drought seems to have had less of a influence on richness than abundance, so this "hump" appears to be more a result of altitude. However, it is also worth noting the much greater than expected species richness estimates for sites 11, 13, and 16.

Water temperature: As with abundance there was no clear relationship between Chao 1 species estimates and water temperature (Figure 100).

pH: Again there was no clear relationship between Chao 1 species estimates and pH (Figure 101).

Stream size: Again no relationship is seen between stream size and the Chao 1 species estimates (Figure 102).

Canopy: As with abundance, open canopy sites had higher Chao 1 richness estimates than closed sites (Figure 103), although this difference was not significant ($t = -1.9264$, $p > 0.05$).

Substrate: Again, as with abundance, there was no significant difference in Chao 1 richness estimates among different substrate types (Figure 104).

Human disturbance: Unlike abundance there was no significant difference between disturbed and undisturbed sites in Chao 1 richness estimates (Figure 105, $t = 0.2932$, $p > 0.05$).

4.2.2.2 Observed species counts

Drought: Not surprisingly, given the sample size independence of the observed species count measure of richness, the relationship between drought and observed species ($\rho = -0.8591$, $p < 0.001$; $r^2 = 0.6505$, $p < 0.001$) is very similar to that seen between drought and abundance (Figure 106).

Altitude: Likewise the altitude-observed species count relationship (Figure 107) is similar to that seen for altitude and abundance (Figure 91).

Water temperature: Again the water temperature-observed species count relationship (Figure 108) is similar to that seen for water temperature and abundance (Figure 92).

pH: The pH and observed species count relationship (Figure 109) is similar to that seen for pH and abundance (Figure 93).

Stream size: The stream size and observed species count relationship (Figure 110) is similar to that seen for stream size and abundance (Figure 94) with no clear relationship being seen.

Canopy: Again, as with the canopy-abundance relationship, sites with an open canopy had higher observed species counts than those with a closed canopy (Figure 111), however this difference was not significant ($t = -2.0767$, $p > 0.05$).

Substrate: Again, as with all the previous factors, the relationship between substrate type and observed species count (Figure 112) is essentially a reflection of the substrate and abundance relationship.

Human disturbance: Sites subject to human disturbance had a slightly higher, but non-significant ($t = 1.0831$, $p > 0.05$), observed species count (Figure 113). However, while all but one species was found in undisturbed sites, only six species were found in disturbed sites, with only *S.* sp. B being restricted to disturbed sites.

Table 7 Species counts, estimators and standard errors for the pooled sample.

Estimator	Estimate	Standard Error
Observed count	17	NA
Chao	19.66	4.88
Jack1	21.75	2.53
Jack2	23.69	NA
Boot	19.17	1.45
Chao1	17.06	0.73
ACE	17.37	2.05

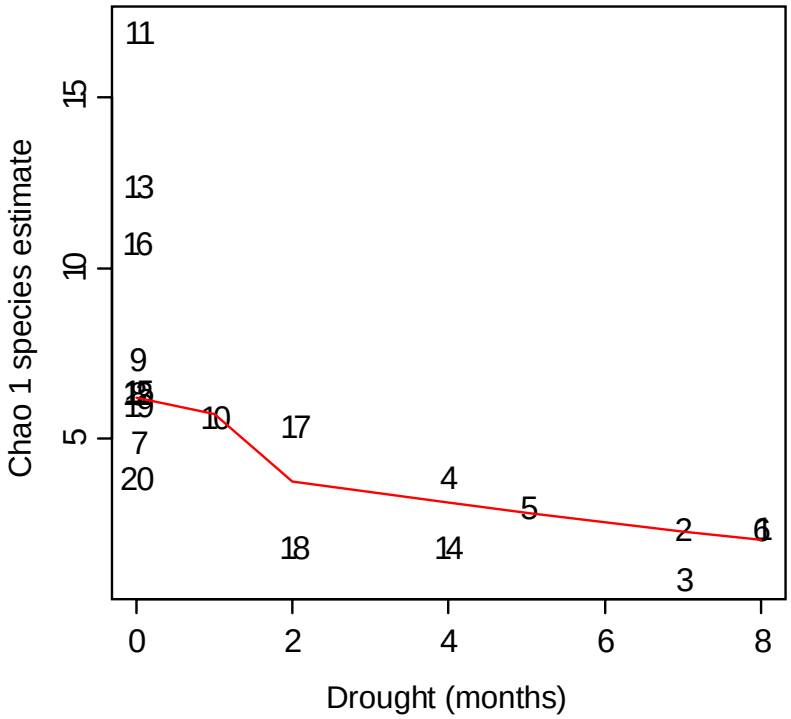

Figure 98 Drought and black fly estimated species

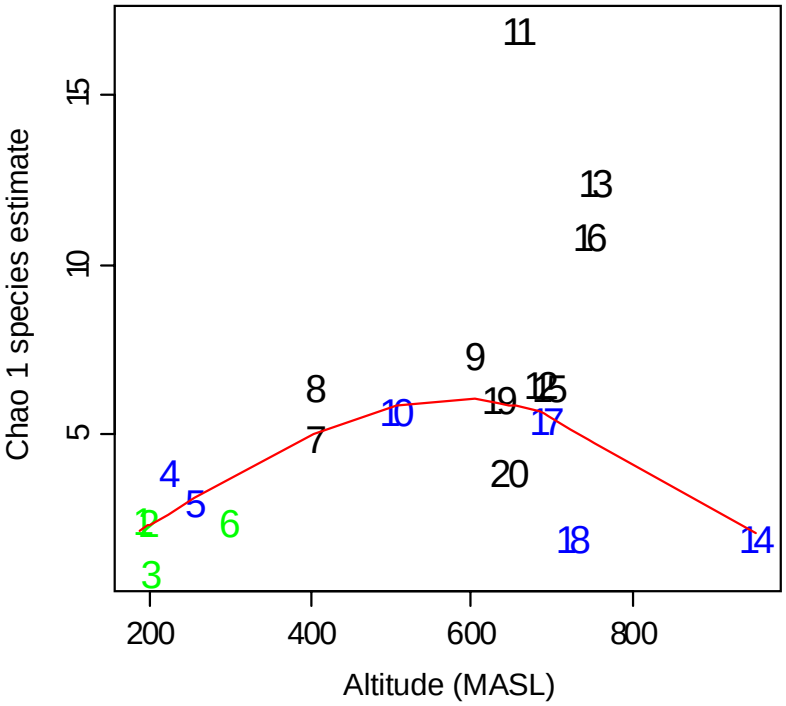

Figure 99 Altitude and black fly estimated species. Black = no drought, blue = less than six months drought, green = six or more months drought

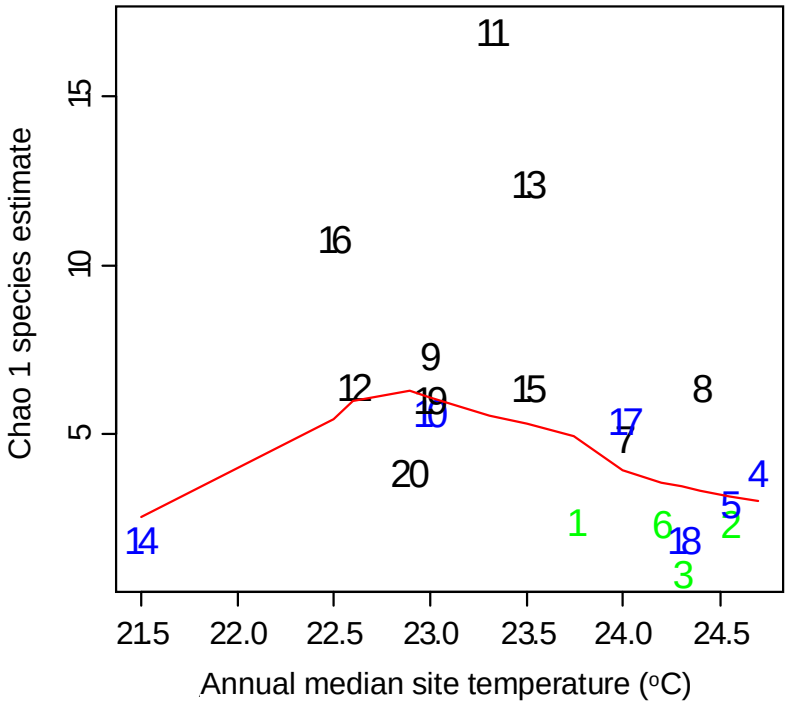

Figure 100 Water temperature and black fly estimated species. Black = no drought, blue = less than six months drought, green = six or more months drought

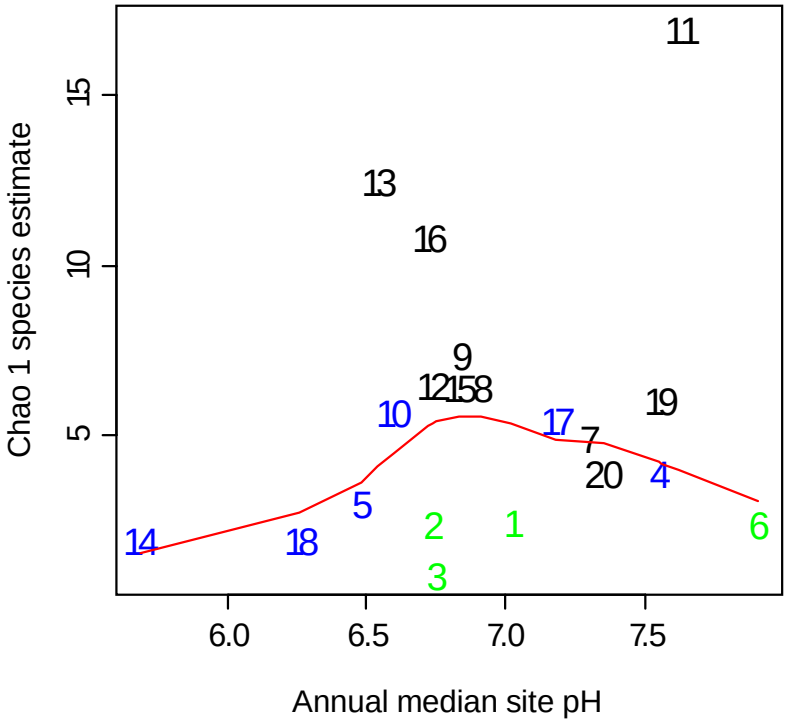

Figure 101 pH and black fly estimated species. Black = no drought, blue = less than six months drought, green = six or more months drought

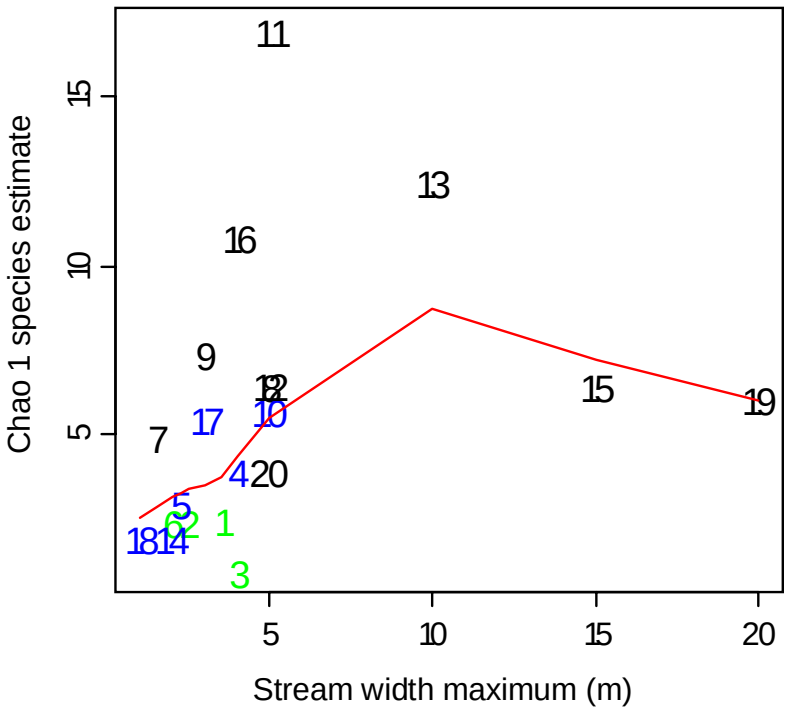

Figure 102 Stream size (max. width) and black fly estimated species. Black = no drought, blue = less than six months drought, green = six or more months drought

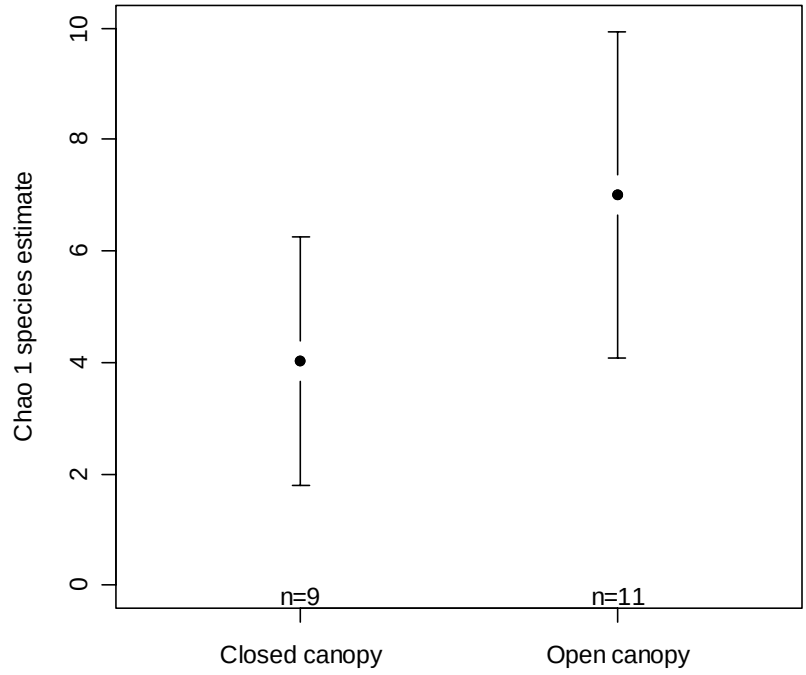

Figure 103 Canopy and black fly estimated species

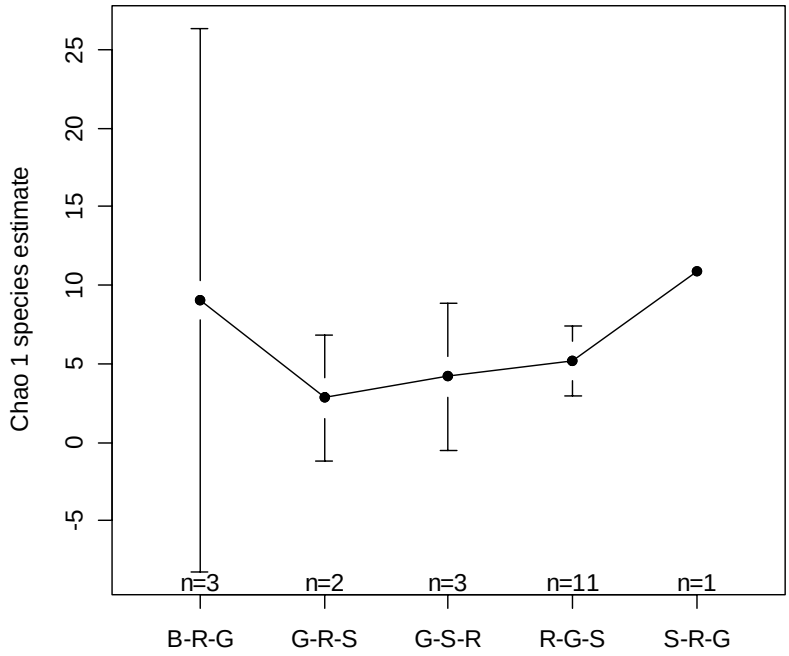

Figure 104 Substrate type and black fly estimated species. B = bedrock, R = rock, G = gravel, and S = sand

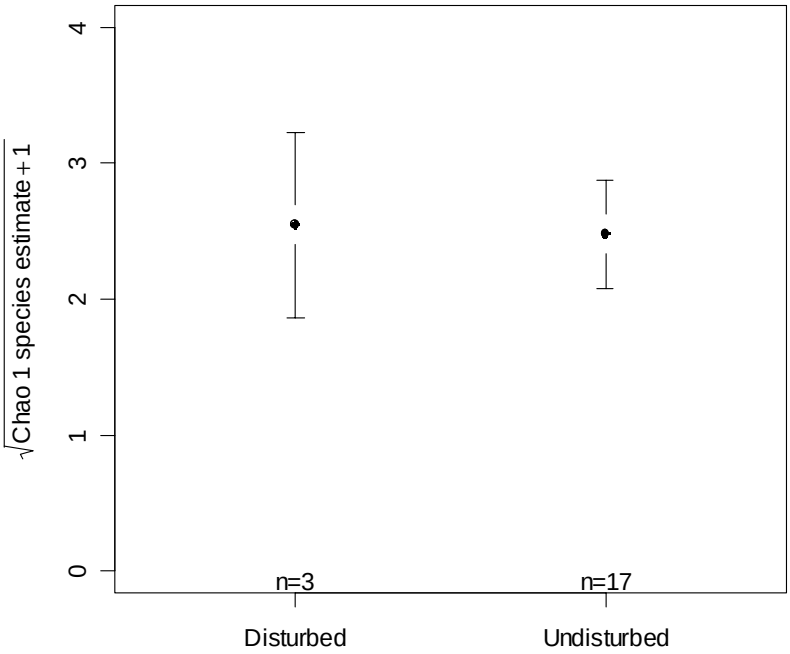

Figure 105 Human disturbance and black fly estimated species

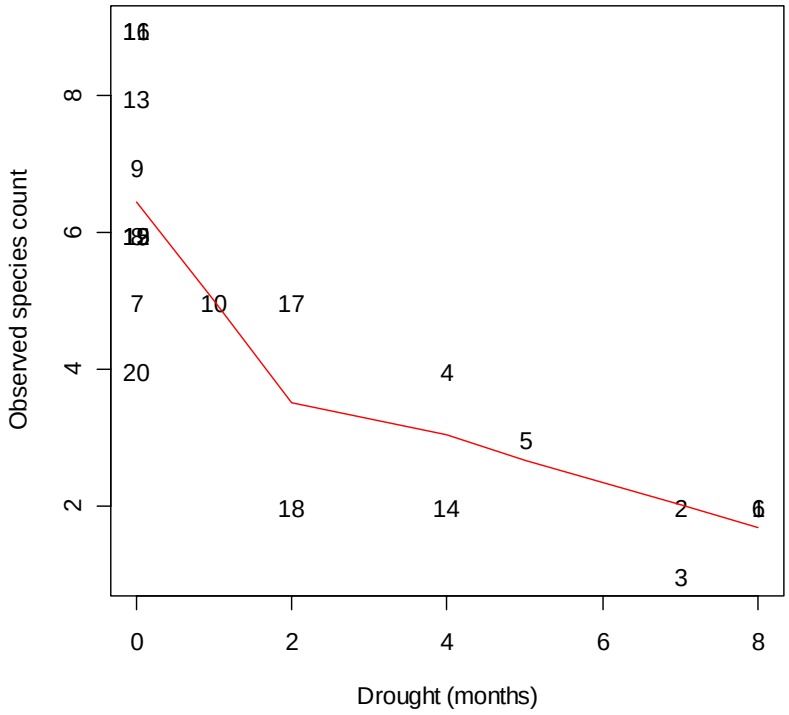

Figure 106 Drought and black fly observed species

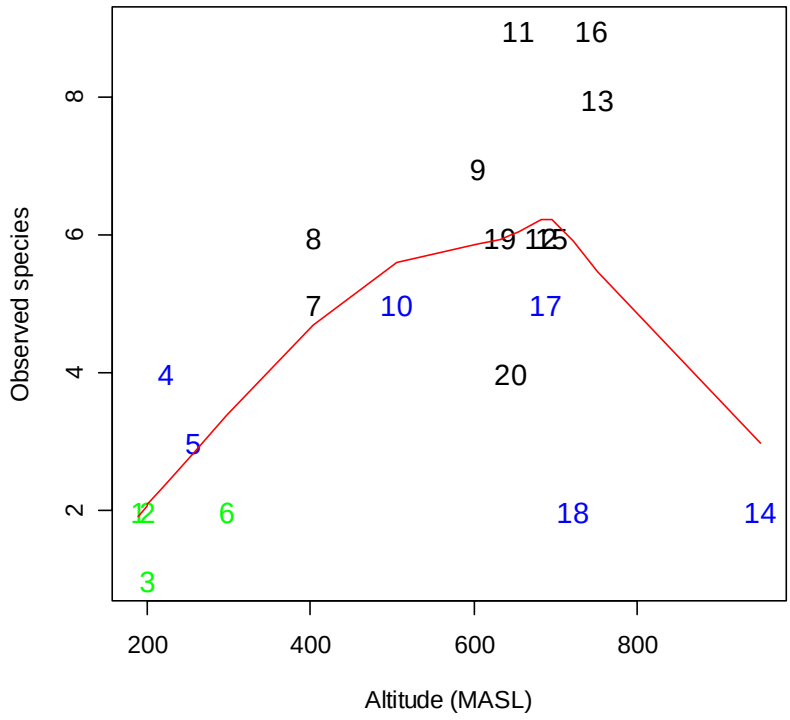

Figure 107 Altitude and black fly observed species. Black = no drought, blue = less than six months drought, green = six or more months drought

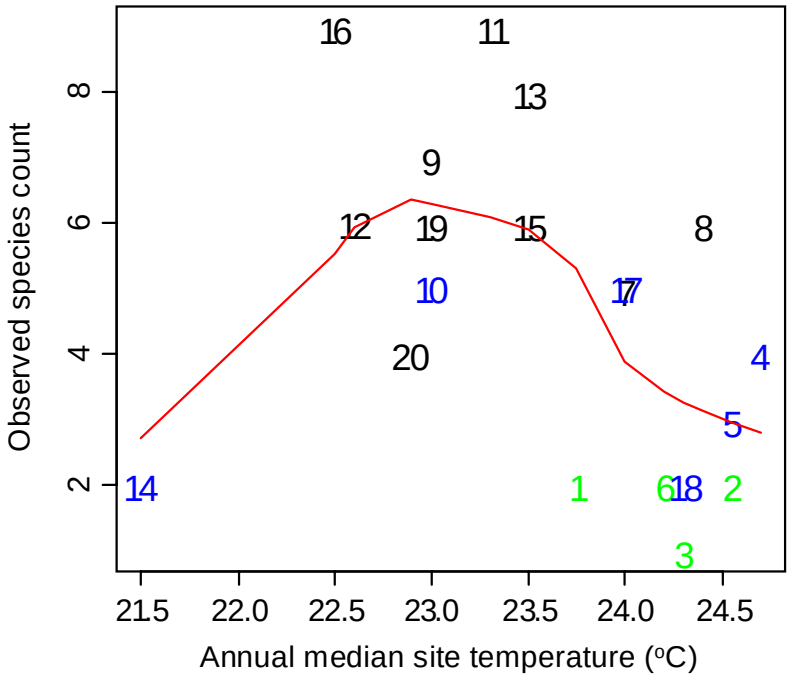

Figure 108 Water temperature and black fly observed species. Black = no drought, blue = less than six months drought, green = six or more months drought

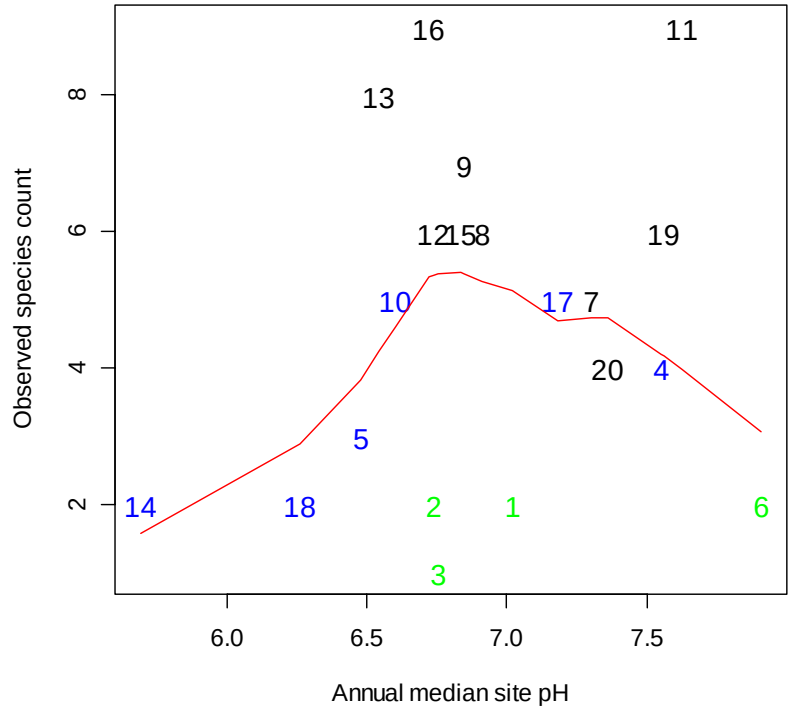

Figure 109 pH and black fly observed species. Black = no drought, blue = less than six months drought, green = six or more months drought

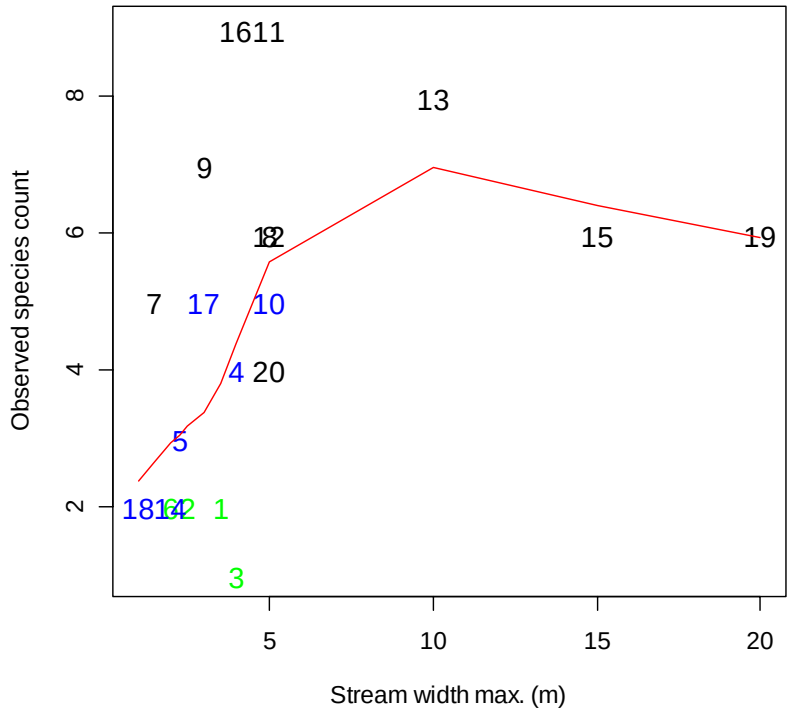

Figure 110 Stream size (max. width) and black fly observed species. Black = no drought, blue = less than six months drought, green = six or more months drought

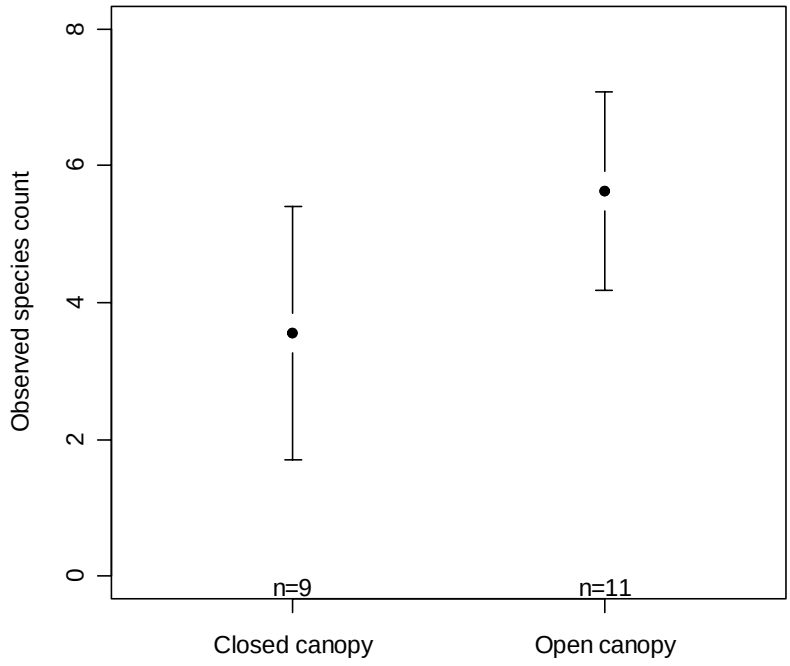

Figure 111 Canopy and black fly observed species

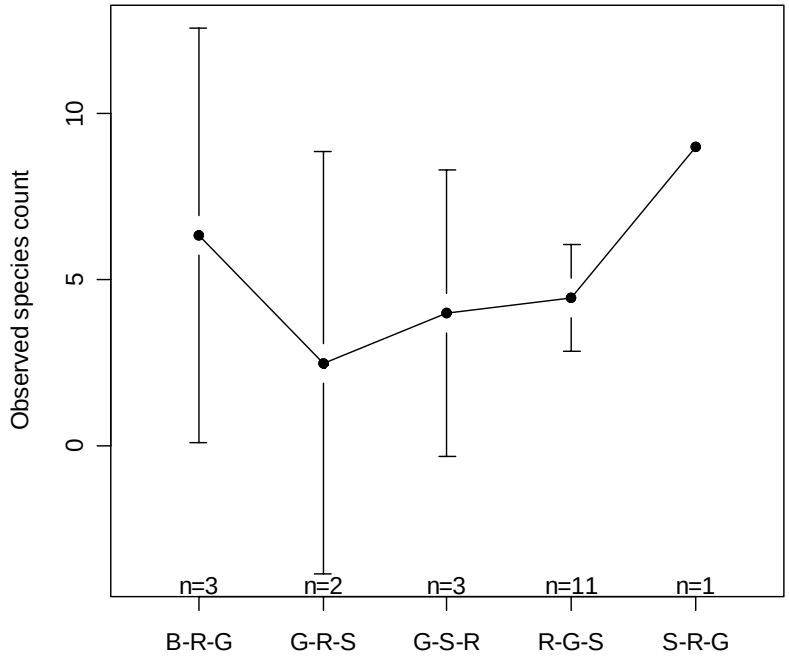

Figure 112 Substrate type and black fly observed species. B = bedrock, R = rock, G = gravel, and S = sand

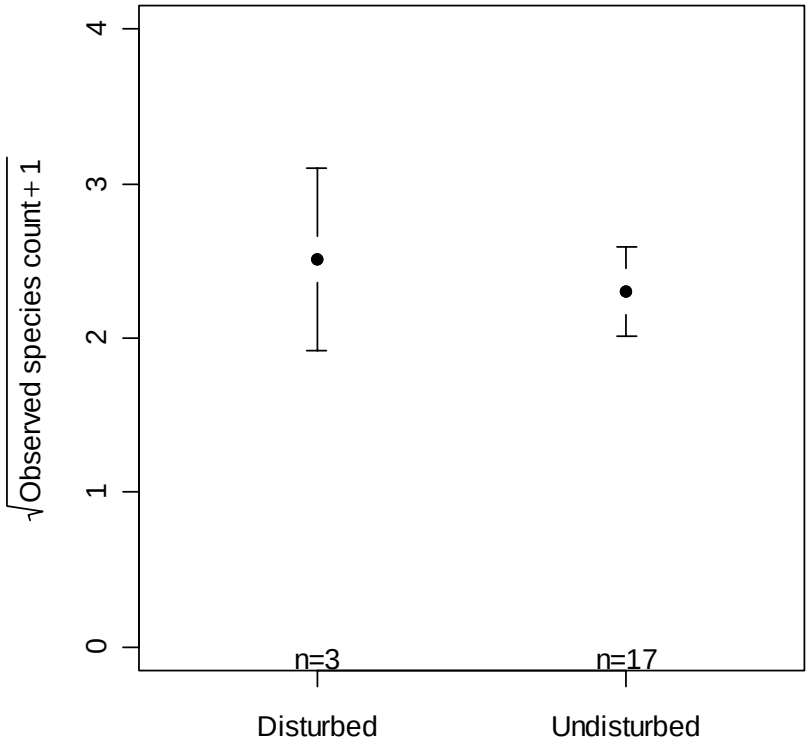

Figure 113 Human disturbance and black fly observed species

4.2.3 Fisher's α

Drought: As with other measures drought had a large influence on a site's Fisher's α diversity index (ρ = -0.7299, $p < 0.001$; $r^2 = 0.4618$, $p < 0.01$; Figure 114) though there was great variation among sites not experiencing drought.

Altitude: The relationship between altitude and Fisher's α was similar to that seen for the other parameters examined, with a generally increasing trend with altitude. However in large part this is probably a result of drought effects which probably also result in the low Fisher's α of site 14 (Figure 115).

Water temperature: The relationship between water temperature and Fisher's α was essentially the inverse of the altitude and Fisher's α relationship (Figure 116), something perhaps not unexpected given the strong inverse relationship between altitude and water temperature.

pH: As with other parameters there was no association between Fisher's α and pH (Figure 117).

Stream size: Again, as with other parameters there was no association between Fisher's α and stream size (Figure 118).

Canopy: There was no significant difference in Fisher's α between sites with open and closed canopies (Figure 119, $t = -1.1808$, $p > 0.05$), though as with other parameters open canopy sites had higher values.

Substrate: There were no significant differences in Fisher's α among the different substrate types (Figure 120).

Human disturbance: There was no significant difference in Fisher's α between disturbed and undisturbed sites (Figure 121, $t = -0.2533$, $p > 0.05$).

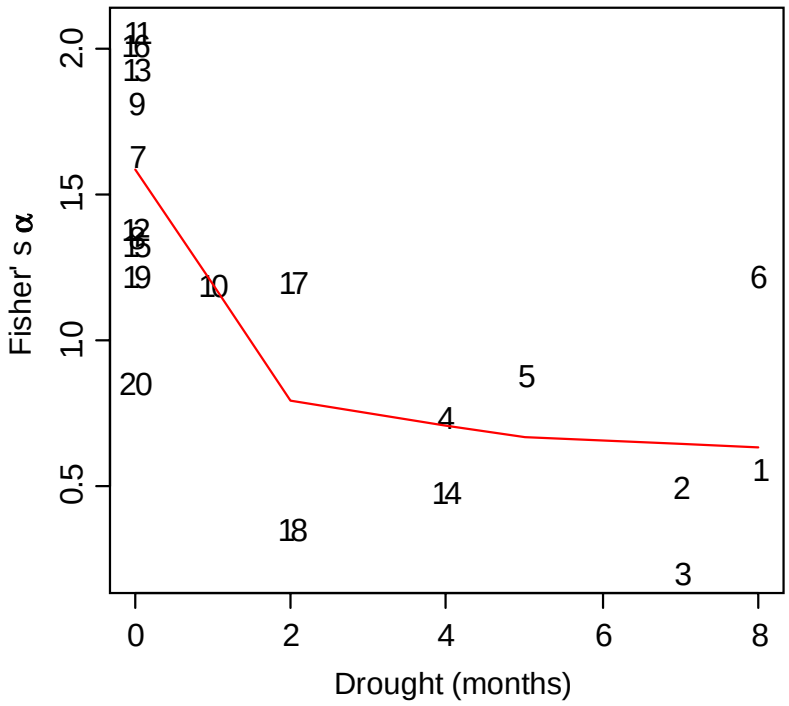

Figure 114 Drought and black fly Fisher's α

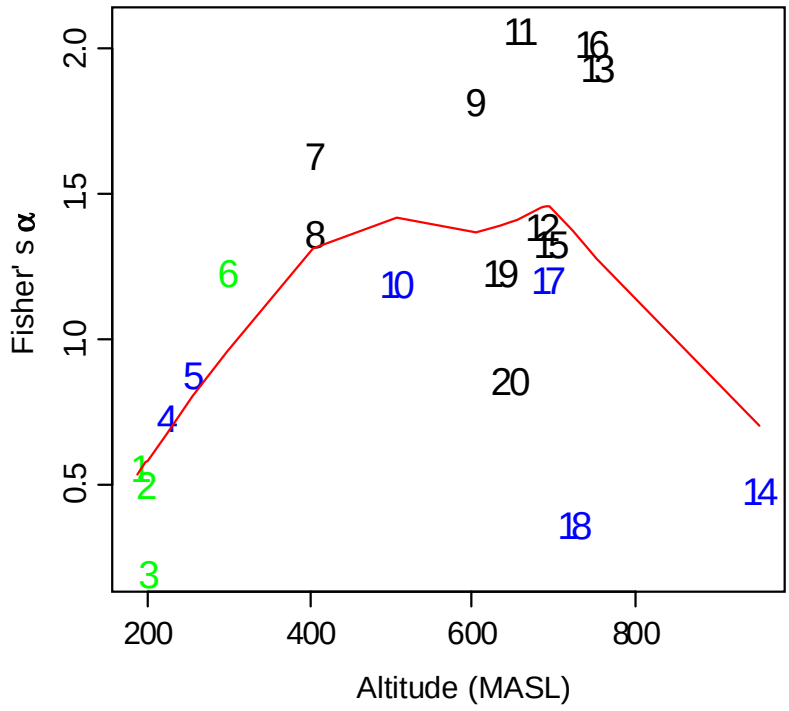

Figure 115 Altitude and black fly Fisher's α. Black = no drought, blue = less than six months drought, green = six or more months drought

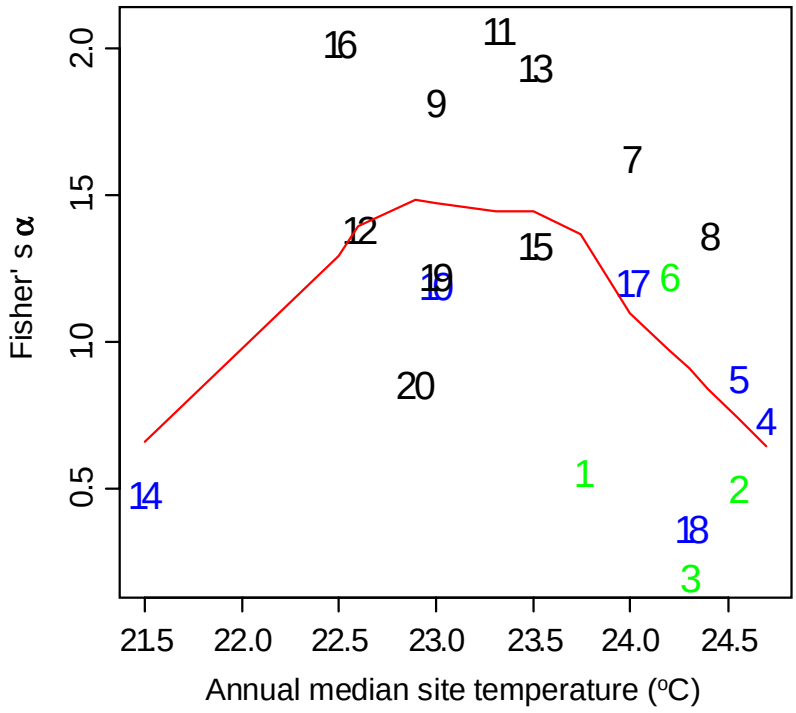

Figure 116 Water temperature and black fly Fisher's α. Black = no drought, blue = less than six months drought, green = six or more months drought

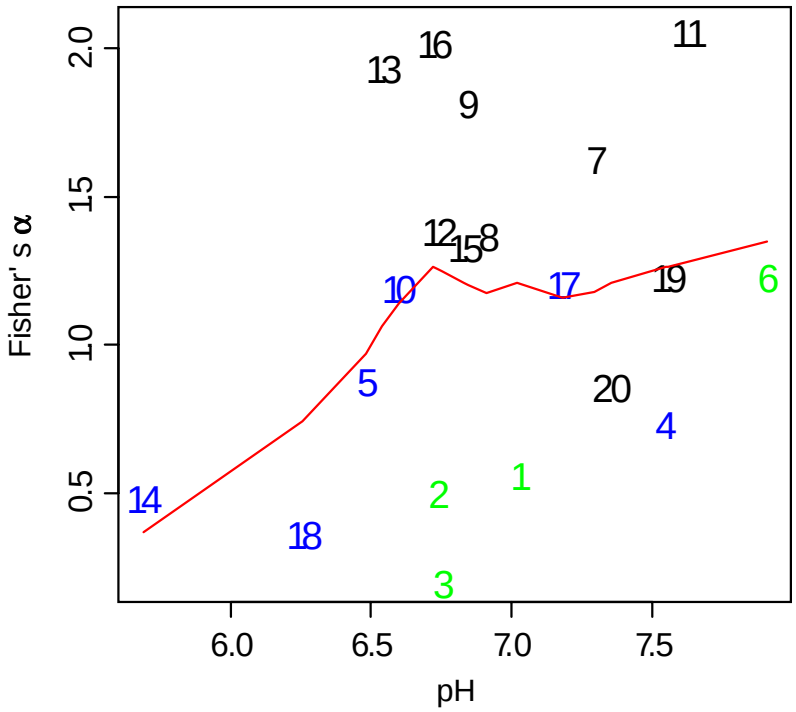

Figure 117 pH and black fly Fisher's α. Black = no drought, blue = less than six months drought, green = six or more months drought

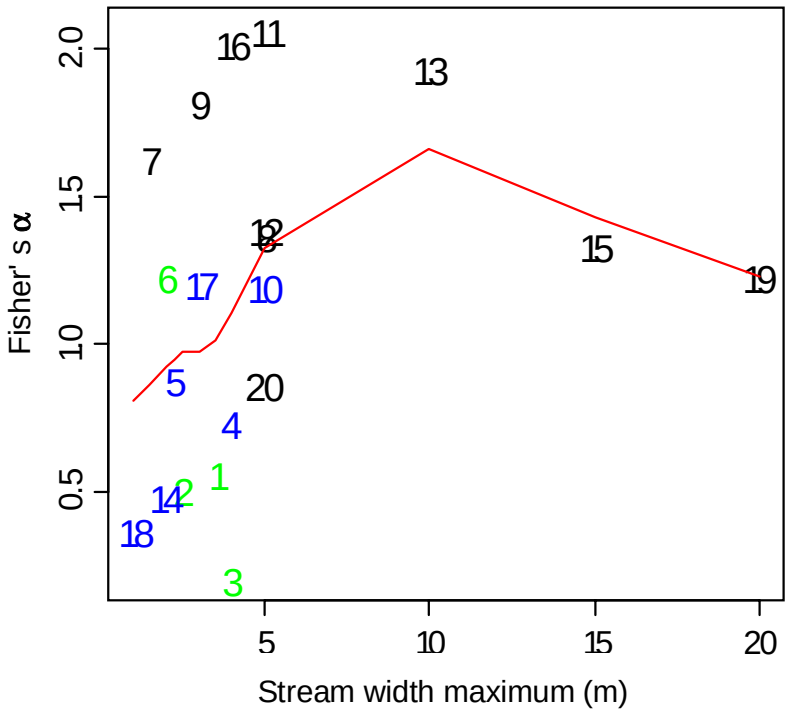

Figure 118 Stream size (max. width) and black fly Fisher's α. Black = no drought, blue = less than six months drought, green = six or more months drought

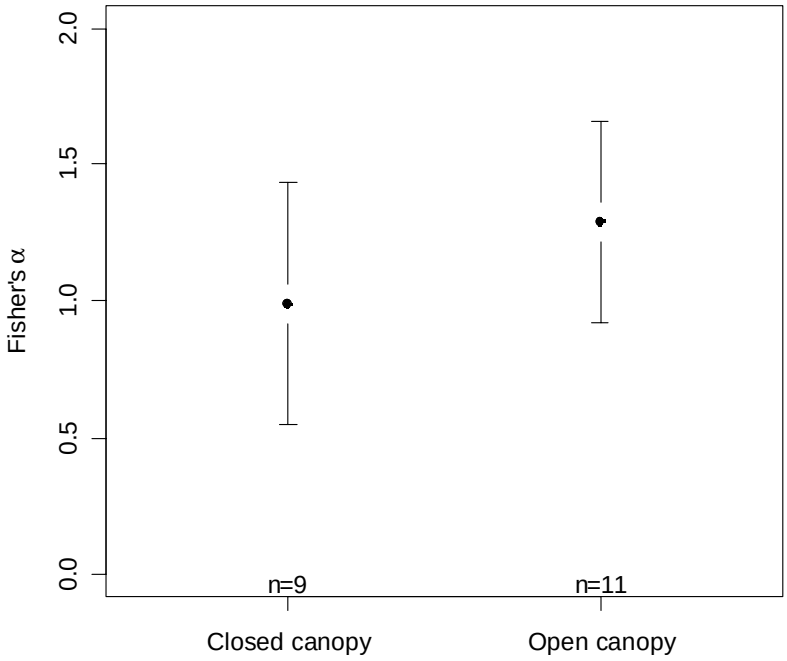

Figure 119 Canopy and black fly Fisher's α.

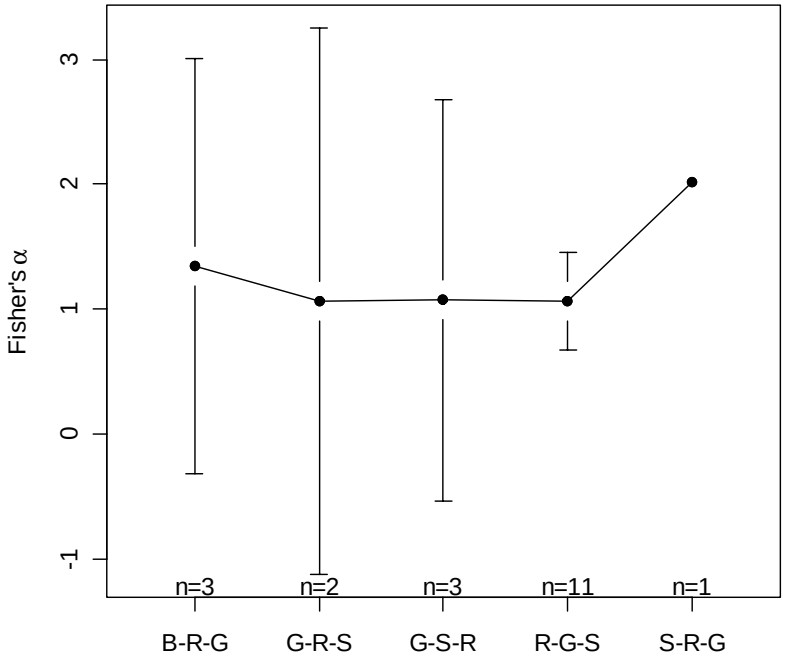

Figure 120 Substrate type and black fly Fisher's α. B = bedrock, R = rock, G = gravel, and S = sand

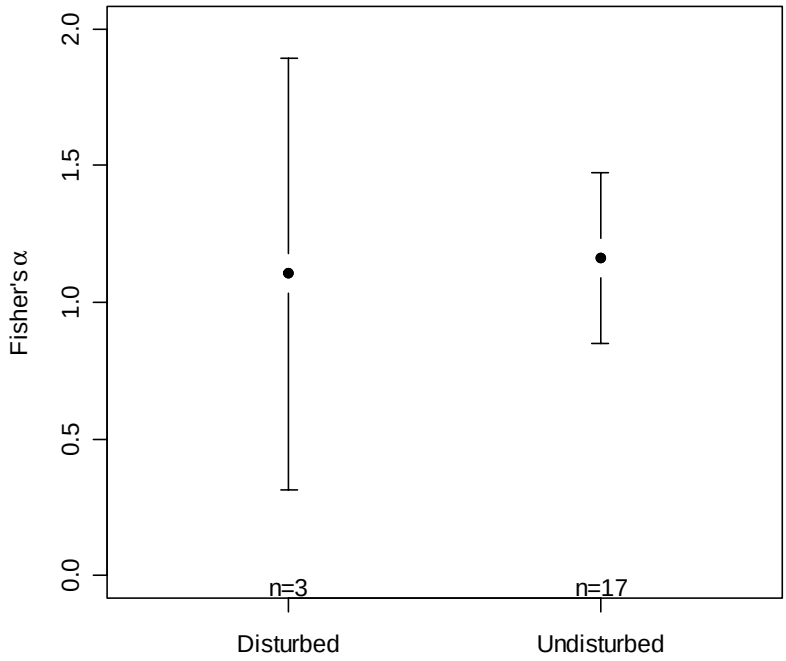

Figure 121 Human disturbance and black fly Fisher's α.

4.2.4 Community composition

The most prominent features of the unconstrained ordination (Figure 122) were the distinctness of sites 14 and 18 which were both very distinct from the main cluster as well as each other. Site 18's distinctness was largely the result of its low richness (2 species, *Simulium sheilae* and *S. siamense*) with *S. sheilae* dominating (≈ 95%). *Simulium sheilae* was unusual in that it was only found at 4 sites with ≈ 86% of the catch from site 18. Site 14 similarly had only two species (*S. angulitylum* and *S. feuerborni*), with *S. angulitylum* dominant (≈ 79%). However, while *S. angulitylum* was dominant at site 14, it was also a common species at all sites except site 18. Another aspect of the site 14 fauna was the occurrence of *S. feuerborni* which was only found at this site. Aside from these two outlying sites the ordination shows a rather broad cluster based upon the ordination origin though there are also indications of small clusters (cluster 1 = sites 4, 8, 11, 13, 15, 19; cluster 2 = 6, 7, 9, 10, 12, 16, 17, 20; cluster 3 = 1, 2, 3, 5) mainly separating on the second axis within this cloud.

The distinctness of sites 14 and 18 (and the species associated with them) were again clear in the constrained ordination (Figur 123). Of the physical variables examined none were clearly indicative of any cluster and only one was clearly associated with an ordination axis. Drought was closely aligned to axis 1, this being the primary axis differentiating site 14 (with *S. feuerborni*) and also the cluster of sites 1, 2, 3 and 5 (with drought) from site 4, the other low altitude site being marked by lack of drought.

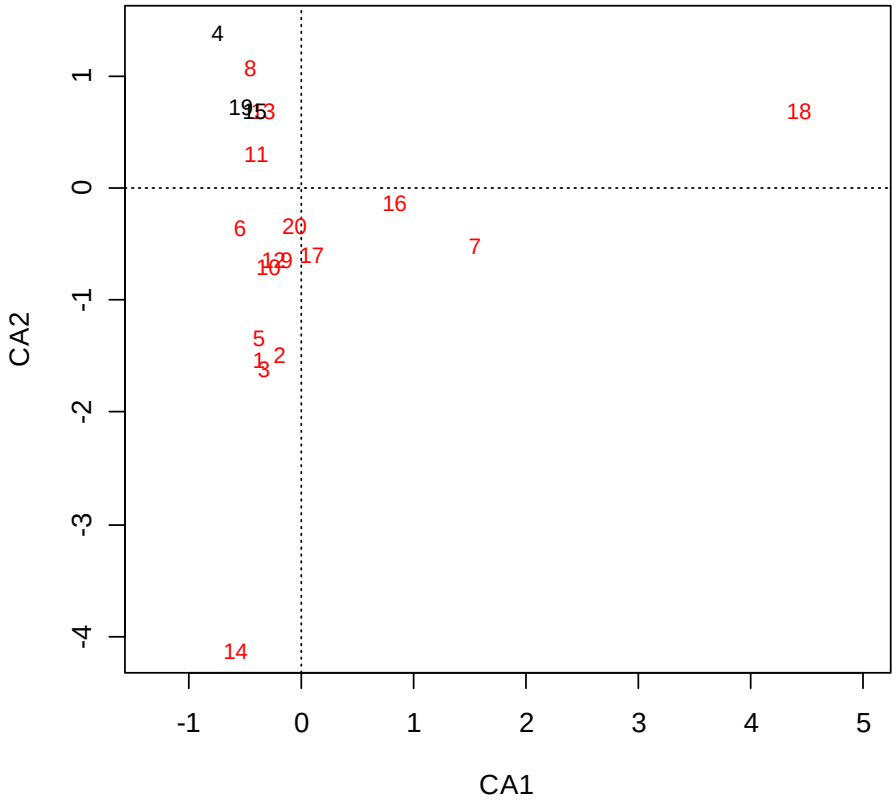

Figure 122 Transformed Black fly CCA (without constraint) ordination diagram and the 20 collection sites. Black = disturbed, Red = undisturbed

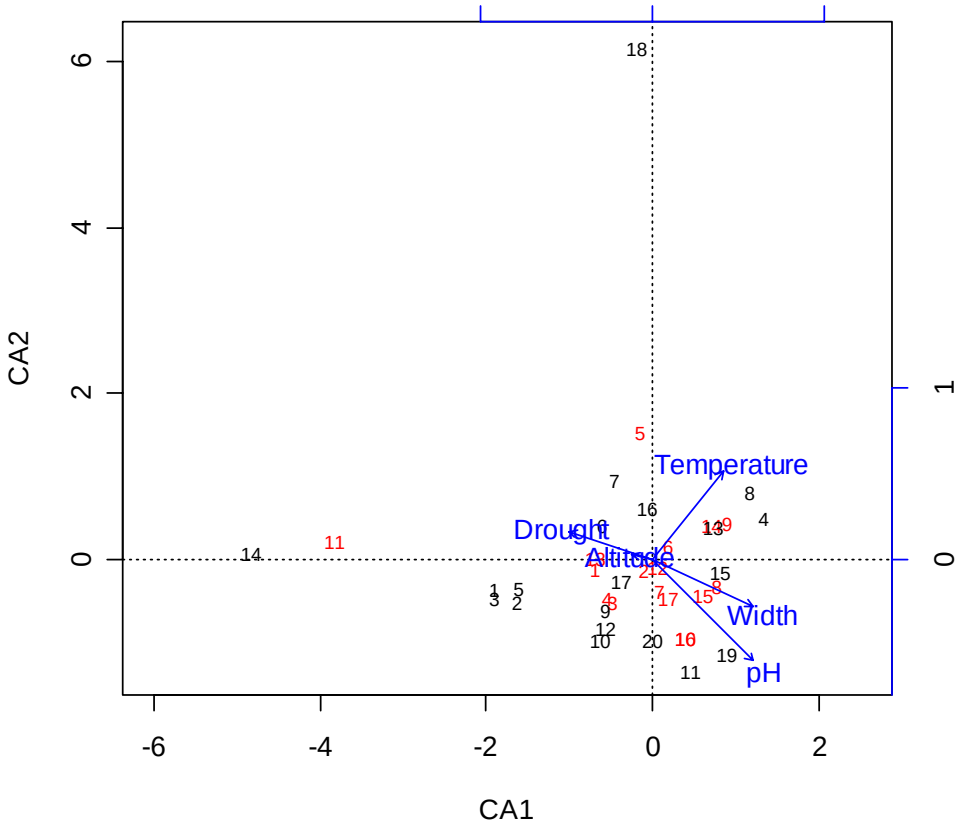

Figure 123 Black fly CCA ordination diagram and the 20 collection sites. Black = collection sites, Red = species

5 Other Families

In addition to the Simuliidae, ten other families of Diptera were recorded from Khao Yai National Park, namely the suborder Nematocera: Tipulidae, Blephariceridae, Psychodidae, Dixidae, Ceratopogonidae, and Chironomidae and the suborder Brachycera: Tabanidae, Athericidae, Ephydridae, and Muscidae. The general characters and larval habitats of each family are shown below. A list of all lotic Diptera and their occurrence in this study, are shown in Appendix Table 1.

1. Tipulidae (crane flies)

Larvae differ from other Nematocera by having only the anterior portion of head capsule heavily sclerotized, the posterior portion of head being incomplete and retracted into thorax. Three genera of this family were identified in this study including *Antocha*, *Hexatoma* and *Tipula* (Figure 124). *Antocha* larvae were found from fallen leaves and bedrock in fast current while *Hexatoma* and *Tipula* were collected from in fallen leaves and sand in slower current. Dudgeon (1999) noted that the Oriental contains around thirty-three genera. He also gave a summary of the larval habits of some tropical Asian genera.

2. Blephariceridae (net-winged midges)

Larvae are dorsoventrally flattened forms and divided into six distinct body divisions, with a sucker disc ventrally on each. The head and thorax are fused with abdominal segment 1 to form the first body division. There were two genera from this study, *Blepharicera* and *Apistomyia* (Figure 125). Both were collected from bedrock while *Blepharicera* also occurred from the rocks. The Oriental Region contains around nine genera (Dudgeon, 1999).

3. Psychodidae (sand flies or moth flies)

Larvae are recognized by absent of prolegs and the secondary annulations on thoracic and abdominal segments, which may have dorsal sclerites on many annuli. Ventral suckers are present in some taxa. *Neotelmatoscopus* and *Telmatoscopus* were found in this study (Figure 126). The latter was found on fallen leaves while *Neotelmatoscopus* occurred on fallen leaves, rocks, and trailing leaves.

4. Dixidae (meniscus midges)

Larvae have two pairs of ventral prolegs on each of the first and second abdominal segments and the body terminates in two pairs of fringed processes. This family was found from fallen leaves.

5. Ceratopogonidae (biting midges)

Larvae have usually well-developed, sclerotized head capsule, but lack prolegs. Most of aquatic species has the body divided into beadlike segments. There was only one sample collected in this study from sand.

6. Chironomidae (non-biting midges)

Larvae have the anterior and posterior pairs of prolegs bearing apical hooks. Head capsule is well sclerotized. They were found from rocks, bedrock, fallen leaves, and trailing leaves.

7. Tabanidae (horse flies or deer flies)

Larvae are recognized by a girdle of at least six pseudopods around each segment. There was only one sample, which was from fallen leaf.

8. Athericidae (snipe flies)

Larvae have paired ventral abdominal prolegs on the first seven abdominal segments and a single ventral proleg and a pair of fringed caudal projections on the eighth segment. They were found from fallen leaves.

9. Ephydridae (shore flies)

Larvae have a pair of respiratory tubes at the end of the abdomen with a dark sclerotized ring around the tip. There was only one sample, which was found from fallen leaf.

10. Muscidae (house flies or aquatic muscids)

Larvae have a pair of short respiratory tubes at the end of the abdomen and the same segment bears a pair of relatively long prolegs that are tipped with a series of small hooks. Prolegs are absent on other abdominal segments thought, creeping welt sometimes are present ventrally. There was only one sample in this study from fallen leaf.

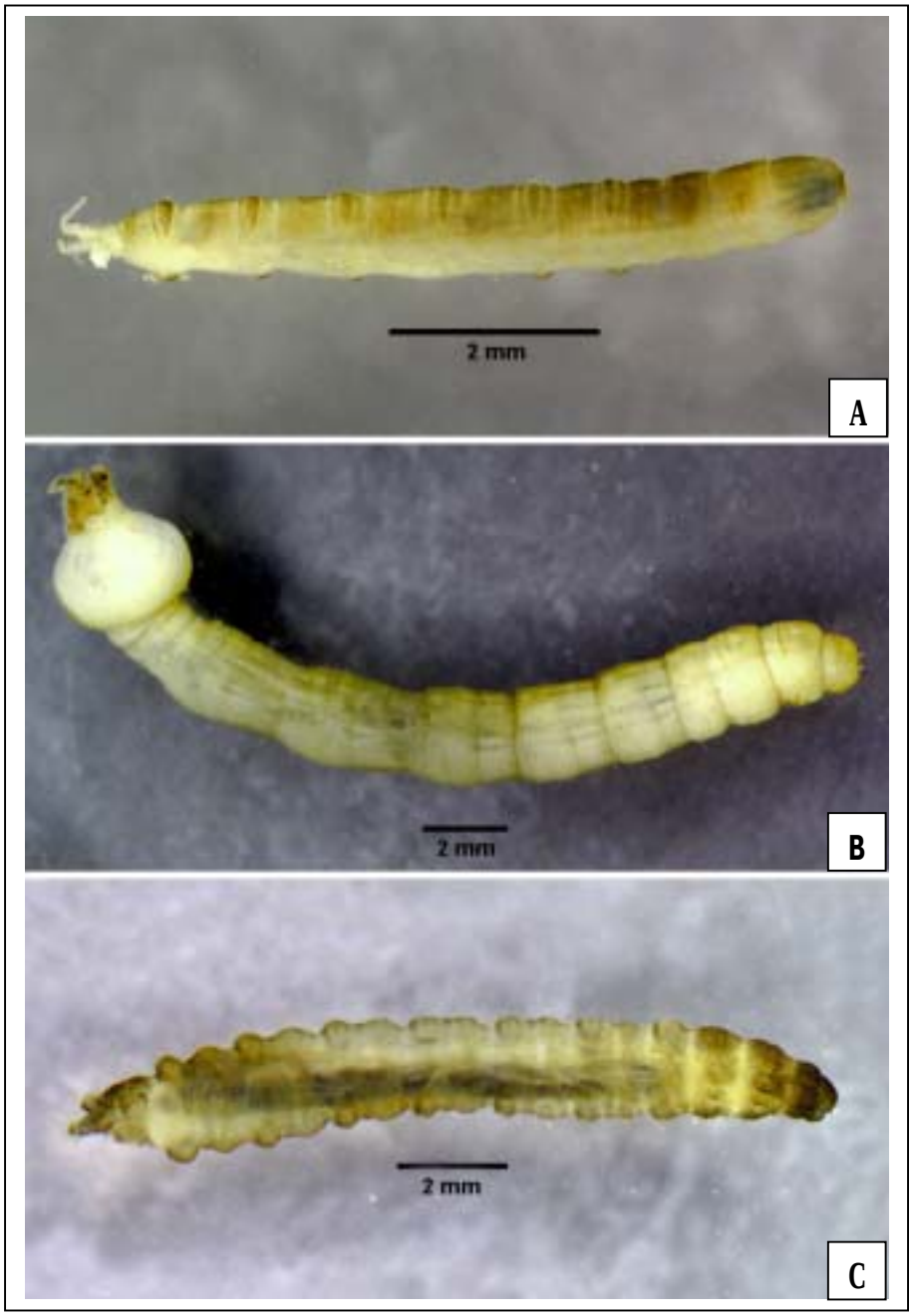

Figure 124 Larval Tipulidae (Diptera) showing lateral view. A, *Antocha* sp.; B, *Hexatoma* sp.; C, *Tipula* sp.

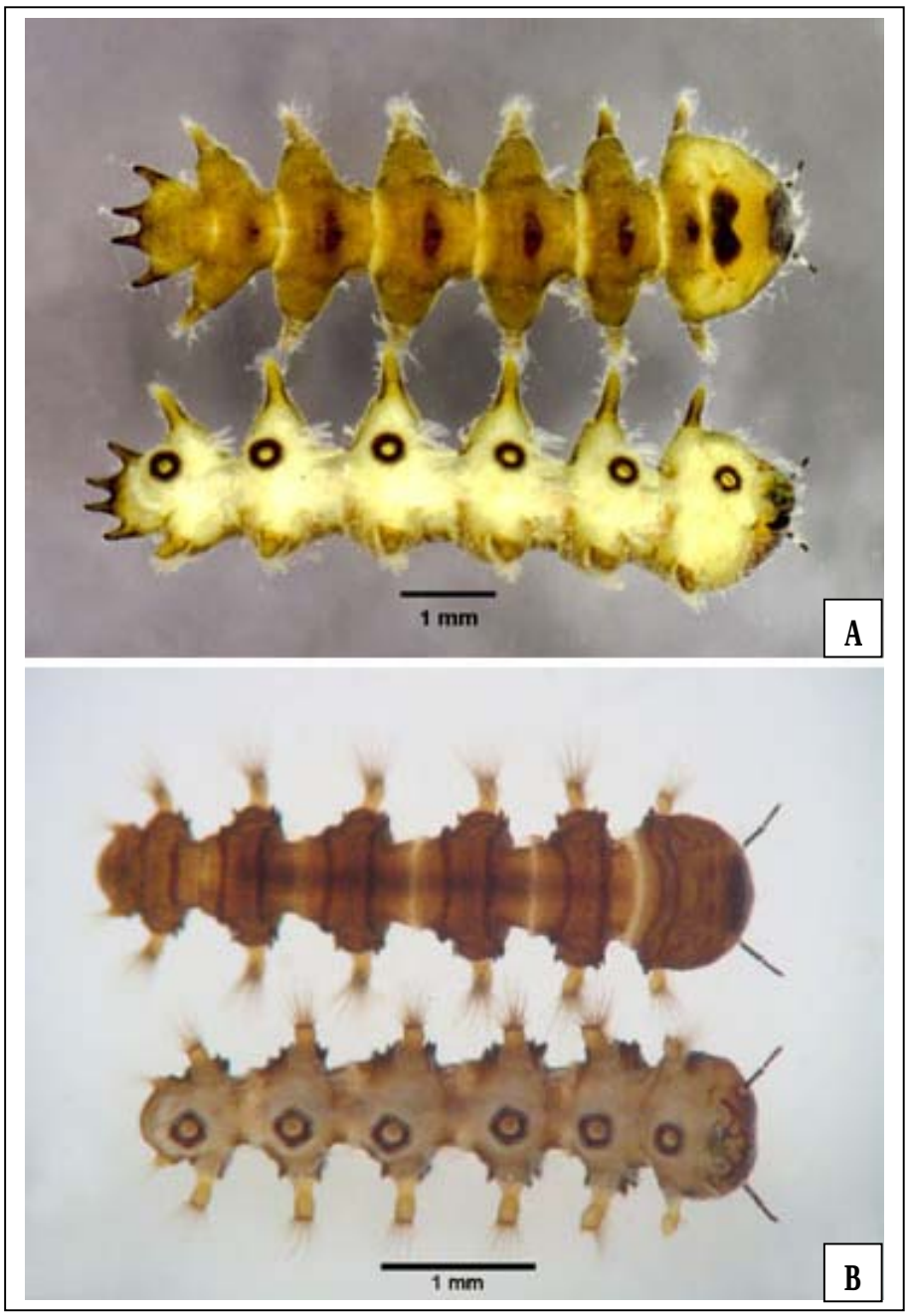

Figure 125 Larval Blephariceridae (Diptera) showing dorsal and ventral view. A, *Blepharicera* sp.; B, *Apistomyia* sp.

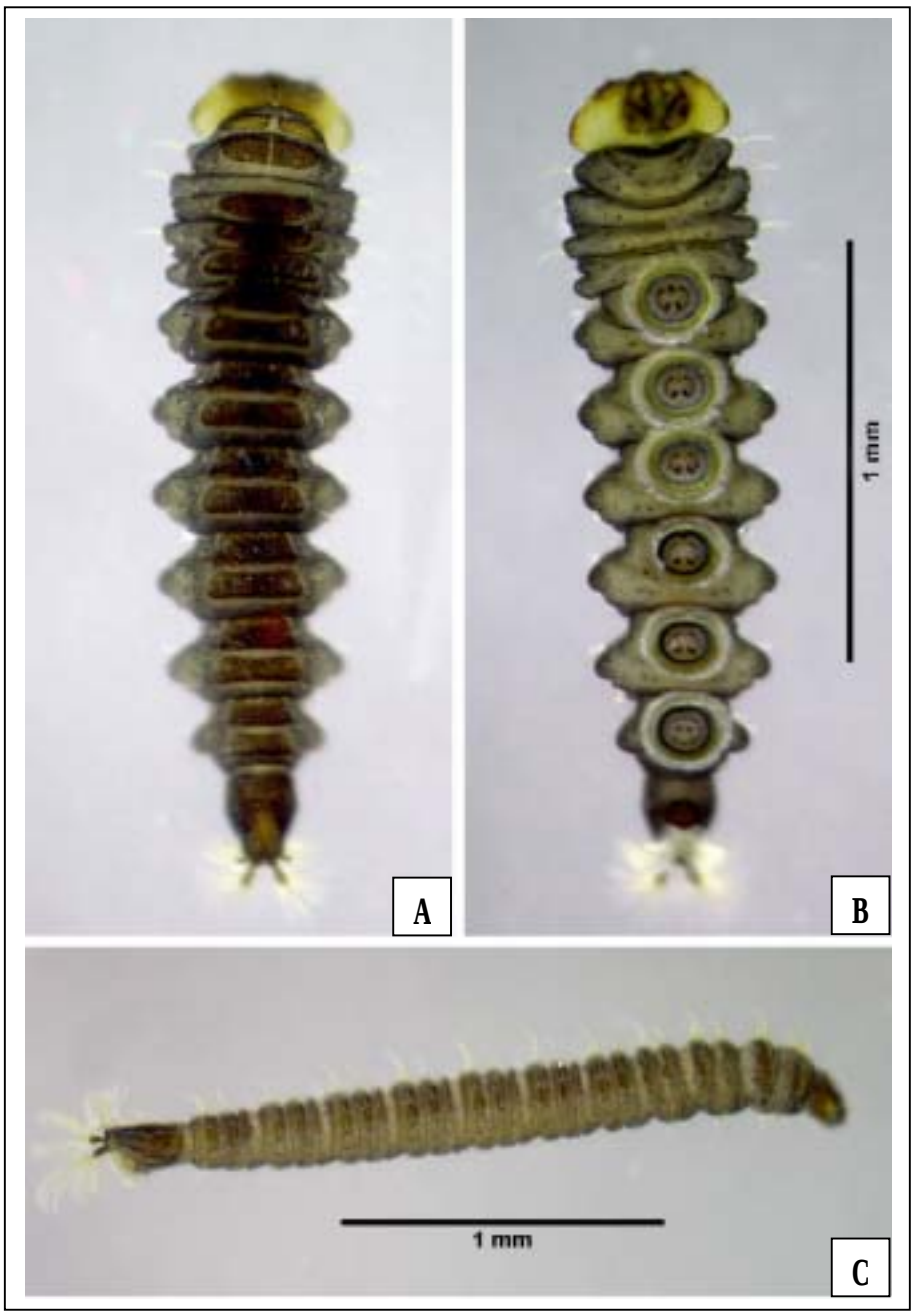

Figure 126 Larval Psychodidae (Diptera) showing dorsal, ventral and lateral view. A, B *Neotelmatoscopus* sp.; C, *Telmatoscopus* sp.

CONCLUSIONS

Of the 22 species of black flies (Diptera: Simuliidae) occurring in streams at Khao Yai National Park, 16, including one species is newly recorded from Thailand (*Simulium novemarticulatum* Takaoka and Davies, 1995), are previously recorded species and 6 represent undescribed species (*S*. sp. A, *S*. sp. nr. *sheilae*, *S*. sp. B, *S*. sp. C, *S*. sp. D, and *S*. sp. E). All species belong to the genus *Simulium* Latreille *s.l.* and three subgenera, *Gomphostilbia* Enderlein (12 spp.), *Nevermannia* Enderlein (1 sp.) and *Simulium* Latreille *s.str.* (9 spp.). Specimens were identified primarily from morphological characters of mature larvae and pupae, with supplemental characters from chromosomal data of larval salivary glands. For most species, identifications and life stages associations were confirmed by rearing pupae to obtain adults.

Twelve *Simulium* species of the subgenus *Gomphostilbia* were analyzed chromosomally. Major landmarks in the IIS arm were emphasized and compared with the chromosomes of some reference species described by Kuvangkadilok, *et al.* (2003). All larvae had a chromosomal complement of $n = 3$. Positions of landmarks in the IIS arm, specifically the positions of the bulge and the ring of Balbiani, distinguished most species, including some that were morphologically indistinguishable. Species with landmarks in similar positions differed in larval morphology. The combination of chromosomal landmarks in the IIS arm and selected structural features, especially the form of the cuticular setae on the larval abdomen, permitted accurate identification of all species in this study. Chromosomal characters greatly assisted morphological identification of larvae in this subgenus, especially for morphologically similar species such as *S. asakoae*, *S. sheilae*, and *S*. sp. nr. *sheilae*.

Seventeen species had sufficient data to allow assessment of biodiversity and influence of environmental factors. The species accumulation curve for the sampling shows that sampling has been quite effective and close to completion.

The main factor affecting simuliid larva abundance was drought, as indicated by a strong negative relationship between the period of drought a site experienced and

larval abundance as well as the Chao 1 species estimate, observed species counts, and Fisher's α. Simuliid abundance showed a positive correlation between larval abundance and altitude. This relationship is mostly a result of the influence of drought, which also had a relationship with altitude-i.e. lower altitude sites typically experiencing greater drought. The relationship between altitude and the Chao 1 species estimate, observed species counts, and Fisher's α were similar to that seen for altitude and abundance. Abundance, the Chao 1 species estimate, observed species counts, and Fisher's α were not associated with median water temperature, median water pH or stream size. The relationship between water temperature and Fisher's α is essentially the inverse of the altitude and Fisher's α relationship. Sites with an open canopy had significantly higher larval abundance than those with a closed canopy while there was no significant difference in Chao 1 richness estimates, observed species count and Fisher's α between sites with open and closed canopies, though open canopy sites had higher values. There were no significant differences in abundance, Chao 1 richness estimates, observed species count and Fisher's α among the different substrate types. Simuliid abundance was significantly higher in streams subject to human disturbance while there were no significant differences between disturbed and undisturbed sites in Chao 1 richness estimates, observed species count and Fisher's α.

Unconstrained ordination demonstrated the distinctness of sites 14 and 18, which are both very distinct from the main cluster as well as being distinct from each other. Site 18's distinctness is largely the result of its low richness (2 species, *Simulium sheilae* and *S. siamense*) with *S. sheilae* dominating (≈ 95%). Site 14 similarly had only two species (*S. angulistylum* and *S. feuerborni*), with *S. angulistylum* dominant (≈ 79%). Another unique aspect of the site 14 fauna was the occurrence of *S. feuerborni*.

The distinctness of sites 14 and 18 (and the species associated with them) were again clear in the constrained ordination. Of the physical variables examined none were clearly indicative of any cluster and only one was clearly associated with an ordination axis. Drought was closely aligned to the first axis, this being the primary

axis differentiating site 14 with (*S. feuerborni*) and also differentiating the cluster of sites 1, 2, 3 and 5 (with drought) from site 4, the other low altitude site being marked by lack of drought.

Ten other Diptera families were recorded in this study area, namely the nematocerous families Tipulidae, Blephariceridae, Psychodidae, Dixidae, Ceratopogonidae, and Chironomidae and the brachycerous families Tabanidae, Athericidae, Ephydridae, and Muscidae.

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APPENDIX

Appendix 1 Procedures for mounting techniques (S. Sing-asa, personal communication).

1. Place specimen into vial with 10% KOH for 2-3 hours or over night (room temperature) or place specimen into vial with 10%KOH at 62-65°C for 10 minutes. (Keep the vial with the acid corked!)

2. Observe vial under scope to ensure that full clearing has occurred.

3. Place specimen in a vial of distilled water (room temperature).

4. Drain off the distilled water and replace it with distilled water twice.

5. Place specimen in a vial of 50% EtOH for about 10 minutes. Move specimen up to 60% EtOH for 10 minutes. Repeat with 70% EtOH, 80% EtOH, 90% EtOH. and 95% EtOH.

6. Place specimen into vial with normal butylalcohol for 10 minutes.

7. Place specimen into vial with 1:1 solution of normal butylalcohol and Xylene for 10 minutes.

8. Place specimen into vial with Xylene for 10 minutes.

9. Carefully dissect the appropriate part using scalpel or dissecting needles.

10. Place one drop of permount in the middle of a microscope slide.

11. Place specimen into permount and apply a coverslip for observation. Work quickly!

Appendix 2 Procedures for staining polytene chromosomes of larval black flies (P.H. Adler, personal communication).

1. Place live larvae in Carnoy's fixative (1 part glacial acetic acid: 3 parts absolute ethanol, with no more than 1 larva per ml of fixative. Decant the fixative and replace it with fresh fixative at least once immediately after making the collection and again an hour later.

2. In a watch glass of Carnoy's, select larvae with well-developed white histoblasts. Slit open the posteroventral portion of the abdomen, using dissecting needles or insect pins.

3. Place larvae in a vial of distilled water for 20 minutes (room temperature).

4. Remove larvae from distilled water and very quickly roll them on bibulous paper to remove the jellied matter from the silk glands, but do not allow the larvae to desiccate.

5. Place larvae in a 1N solution of HCl that has been preheated to 62-65°C. Larvae should be in the HCl at 62-65°C for 10 minutes. (Keep the vial with the acid corked!)

6. Place larvae in a vial of Feulgen stain for about 1 hour.

7. Replace the Feulgen stain with sulfur water and allow the larvae to remain in the sulfur water for about 10 minutes.

8. Drain off the sulfur water and replace it with cold tap water twice. Refrigerate the material until ready to examine the chromosomes, but use within 5 days.

9. Place one drop of 50% acetic acid in the middle of a microscope slide. Place a larva in a drop of cold tap water on one side of the slide. Pull out one salivary

gland and one gonad from the posterior portion of the abdomen. Using needles, macerate the salivary glands in the acetic acid and remove all large pieces of tissue. Work quickly!

10. Apply a coverslip and press on it with firm thumb pressure under a piece of bibulous paper.

11. The slide can be viewed to take photograph for not exceed about 1 hour or place one drop of 2% filtered carmine at the edge of the coverslip to keep the preparation from drying out. The slidemount is now temporary and can be viewed for about 1 hour.

12. To make the slide permanent, place it coverslip down on dry ice for a least 1-hour.

13. Pop off the coverslip with a razor blade, and immediately plunge the slide into a petri dish with absolute ethanol for 30 seconds.

14. Remove the slide from the ethanol, blot the edge of the slide only, and quickly apply a small drop of mounting medium (e.g., Euparal®).

15. Place the slide in a dark place to dry. Expect about 25% shrinkage, with fading over time.

Directions for preparing Feulgen Stain

1. Add 2 grams of basic fuchsin powder to 400 grams of distilled water that has first been heated to 80°C (in flask in microwave). Mix well by swirling the flask.

2. Cool the solution to 60°C and add 4 grams of potassium metabisulphite. Mix well by swirling the flask.

3. Cool the solution to 50°C and add 20 ml of 1N HCl. Mix well by swirling the flask.

4. Place parafilm over the top of the flask and place the flask on the counter for 24 h. The fuchsin solution will, however, still retain a color varying from straw to brown due to dissolved impurities.

5. The next day (24 h later), 3 grams of activated charcoal powder should be added to the solution and shaken thoroughly. Quickly filter the solution. The resultant filtrate should be quite without color and indistinguishable in appearance from water. Put this solution in dark plastic bottles and refrigerate. When handling the charcoal powder, be extramely careful not to spill any. It will stain miserably!

Directions for preparing Sulfur Water

1. Add 2 grams of potassium metabisulfite to 400 ml of distilled water.

2. Swirl the container to mix the solution, but be careful not to get solution around the ground glass stopper. (It will freeze the stopper).

3. Finally, add 20 ml of 1N HCl, and quickly stopper the bottle of solution to avoid escape of SO_2. All preparation is done at room temperature.