

# Familiar Versus Cryptic Substrate Preference of Odonata Anisoptera Nymphs in Douglas Lake

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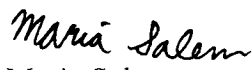
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**Abstract.** Organisms that are subject to predation must evolve strategies to avoid predators or face extinction. Those that lack the physiology to escape at high speeds or employ physical defenses must rely on methods such as burrowing or the use of camouflage. Dragonfly nymphs, unlike their brightly-colored adult counterparts, are dully colored and must attempt to blend in with the aquatic substrate which they inhabit. Because they display cryptic coloration, dragonfly nymphs show certain preferences and aversions to different substrate types based on where they were reared and where their ability to avoid predators is greatest. This study aimed to determine the relative contributions of crypsis and habituation in nymph substrate preference. We found that familiarity played an equal, if not greater, role than crypsis in Odonate nymph substrate choice. Further studies are required to refine this understanding of cryptic and habitual behavior.

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Signed,

  
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## **Introduction**

Organisms that do not have robust physical defenses such as large body size, chemical defenses, or aposematic coloration to ward off predators must rely on cryptic behavior to evade predation. Crypsis is defined as the behavior of seeking out substrates for concealment or a change in coloration to match surroundings (Heinen, 1994). Previous research points to substrate choice and cryptic behavior as the primary and most effective defense mechanisms of Odonate nymphs (Allan, 1995).

Dragonflies (or for the purpose of this paper, Odonates) are predatory insects that oviposit on the surface of shallow water and on the tissues of vascular aquatic plants (Matushkina and Lambret, 2011). Dragonflies follow a hemimetabolous life cycle, in which their eggs hatch into nymphs that then go through multiple instar stages before metamorphosing into adults (Stoks and Córdoba-aguilar, 2011). Nymphs of Odonates reside on a variety of aquatic substrates including submerged wood, vegetation, sand and dark debris (Giacomini and De Marco, 2008). These substrates are rich in food sources for the nymphs, such as small crustaceans, mollusks, aquatic insects and small vertebrates (Pritchard, 1964). Additionally, larger Odonate nymphs have been known to cannibalize smaller nymphs (Merritt and Cummins, 1978). Odonate nymphs are also heavily preyed upon and serve as food sources for various fish, birds, turtles and other aquatic life (Mikolajewski and Johannson, 2003). Physiological characteristics such as dark brown coloration, as well as lateral and dorsal abdominal spines, serve as key anti-predator adaptations for Odonate nymphs (Mikolajewski and Johannson, 2003).

In addition to physiological characteristics, behavior aids in predator avoidance. Odonate nymphs display fixed behaviors, which they maintain regardless of predatory presence, while

their reactive responses are only displayed when predators are detected (Pierce, 1988). Fixed behaviors include the use of crypsis against a substrate, small movements of the limbs and swimming short distances. Reactive responses consist of rapid burrowing into substrates and erratic movements made possible by a water-jet shot from the nymph's rectal respiratory cavity (Pierce, 1988). Furthermore, nymphs maintain a close proximity to the substrate on which they were reared, thus limiting the amount of time they are vulnerable to predation (Moum and Baker, 1988). Based on these findings, our paper assumed the substrate that a nymph was collected from to be its "preferred" substrate. We also assumed that the nymphs have spent the majority of their lives on their preferred substrate and are familiar with it.

The purpose of our study was to determine if *Odonata Anisoptera* nymphs, from the families *Libellulidae*, *Gomphidae* and *Aeshnidae* prefer a substrate that they have previously inhabited versus one that makes them more cryptic. Our study examined whether preferred substrate influenced the cryptic behavioral choices of nymphs. We hypothesized that nymphs would display a preference for the substrate from which they were collected, implying that memory and familiarity influenced the nymphs' substrate decisions along with cryptic strategy.

## **Methods**

### *Specimen Collection*

We conducted this study at the University of Michigan Biological Station, a 13,000 acre biosphere reserve in the Pellston area of northern Michigan (Heinen and Vande Kopple, 2003). We collected Odonate nymphs from along the shoreline of South Fishtail Bay in Douglas Lake. Douglas Lake is a multi-depressional lake composed of seven kettle holes formed by glaciers

during the late Pleistocene Epoch (Pratt and Cairns, 1985). We placed each nymph in a nineteen liter bucket labelled with the substrate type from which it was collected.

We collected and examined nymphs from sand, dark sediment, wood and vegetation substrates. Nymphs found on sand substrates were removed from the water and placed in their respective bucket. Nymphs residing on dark sediment and leaf debris were collected using laboratory sieves with three-millimeter mesh. These were used to filter out smaller sediment particles in order to simplify nymph collection. To collect nymphs from vegetative substrates, plants were removed from the water and combed through, as in most cases the nymphs were burrowed into the base of the plant. Wood substrates were removed from the water and occasionally dismantled to extract the nymphs buried within the crevices. The buckets used for collection were filled with lake water and small amounts of the substrate on which the nymphs were found. This was done in order to minimize distress of the nymphs through the duration of the experiments. In total 200 nymphs were collected and used for trials. Eighty nymphs were collected from wood substrates, and forty nymphs each were collected from the three remaining substrates. The nymphs were stored in buckets in a laboratory for at least twenty-four hours before being subjected to trials for acclimation purposes. The ambient temperature ranged from about 17° to 20° Celsius, which is representative of the nymph's natural living conditions. Lighting was controlled in the lab by covering all windows with black plastic. Overhead lighting was turned on at seven am and turned off at nine pm daily to simulate the diurnal cycle at the time the study was conducted.

### *Experimental Methods*

To test for substrate preference, thirty-eight liter glass aquaria were used to mimic the naturally inhabited substrates of nymphs. Sixteen tanks were split into four groups with the four types of substrates from which the nymphs were collected. For each of the four treatment groups, one tank was considered the control and was filled entirely with a single substrate. This was designated as Tank 1 (Figure 1) for the sand treatment, where both halves of the tank were filled with sand. Each control tank served to expose any confounding spatial or environmental factors in the laboratory that would influence the nymphs' directional choices. It is important to note that the substrates used for testing were not boiled or otherwise purified in order to better simulate the natural environment in which the nymphs were found. After half of the trials were completed, the tanks were rotated 180 degrees to further control against directional bias. Opportunities for nymphs to choose their preferred substrate versus the other three substrates in our study were created by splitting three additional tanks down the middle. Sand tanks 2-4 (Figure 1) exemplify a systematic set up where the sand treatment tanks were filled with sand and dark sediment, sand and wood and sand and vegetation. Four tanks for each additional treatment— dark sediment, wood and vegetation— were prepared using the same system of combinations displayed for sand (Figure 1). In addition to substrates, all sixteen tanks were filled with approximately nineteen liters of water taken from Douglas Lake.

Each nymph was only used in a single trial and was then released. All trials were performed during daytime under laboratory lighting. We conducted each trial with four nymphs collected from the same preferred substrate. We placed one nymph in the center of each of the four treatment tanks one inch below the surface of the water (Figure 1). Nymphs were then given five minutes to acclimate and decide on a substrate within the tank where they preferred to hide.

For control tanks, we recorded which side of the tank (left or right) the nymph was found on. For tanks with two substrates, we recorded which of the two substrates the nymph was found on.

### *Statistical Methods*

We conducted a Pearson's Chi-Square test ( $X^2$ ) using SPSS Statistical Software (Version 21) to test for differences in substrate choice across nymphs collected from different substrates. A Pearson's Chi-Square test with a significance level of 0.05 was used because it tests for Goodness of Fit between expected and observed values for two or more categorical variables (Ambrose and Ambrose, 2007). We ran this test against nymphs collected from two different substrates that were then placed in identical conditions. For example, we compared the substrate choices of nymphs that were collected from wood against the substrate choices of nymphs that were collected from sand in an aquarium filled with wood and sand. This comparison was made for every possible combination of nymphs: wood and sand, wood and dark sediment, wood and vegetation, dark sediment and sand, dark sediment and vegetation and sand and vegetation. The critical  $X^2$ -value utilized for this series of tests was 3.84 (Ambrose and Ambrose, 2007).

We also performed binomial tests by hand to determine if the nymphs' substrate choice was random, or if they had a preference for their preferred substrate. A binomial test assesses the probability of a chain of dichotomous events occurring in a row— where one of the two outcomes is called a “success” and the other is called a “failure” (Dodge, 2008). For the purposes of this study, a “success” case was assigned to any trial in which a nymph chose its preferred substrate and a “failure” case was assigned to any nymph that did not choose its preferred substrate. “Success” and “failure” are statistical terms used to differentiate between two outcomes, and did not indicate that either outcome was favored over the other (Dodge, 2008).

For the set of trials conducted with nymphs collected from wood, the upper-limit binomial value and lower-limit binomial value were calculated by hand. The upper-limit probability indicates that nymphs chose substrates based purely on chance, where exactly half of the nymphs chose their preferred substrate and the other half chose a non-preferred substrate. The lower-limit probability represents a situation where all the nymphs chose their preferred substrate.

For the trials conducted on nymphs collected from wood substrates, p-values were also generated from a normal model using the binomial data. According to Utts and Heckard (2012), a binomial model follows a relatively normal distribution when the expected number of binomial “successes” is greater than or equal to ten. Because we conducted twenty trials with nymphs collected from wood substrates, this condition was met and p-values were calculated using a normal model and a significance level of 0.05. Since we only conducted ten trials for each of the other three substrates, the condition was not met and p-values could not be calculated. For this reason, results from sand, vegetative and dark sediment substrates are interpreted as trends and show no statistical significance.

## Results

Table 1. Binomial-normal analysis of nymphs collected from a wood substrate

Conditions of Aquarium	Binomial Value	Upper-limit Binomial Value	Lower-limit Binomial Value	p-value
Wood and Wood (Control)	0.160	0.176	0.000	0.161
Wood and Sand	0.000	0.176	0.000	0.000
Wood and Vegetation	0.005	0.176	0.000	0.005
Wood and Dark Sediment	0.037	0.176	0.000	0.036

Table 2. Pearson's Chi-Square analysis of nymph substrate choice

	Pearson's Chi-Square Value	p-value
Wood nymphs vs. sand nymphs in wood-sand trials	8.438	0.004
Wood nymphs vs. dark sediment nymphs in wood-dark sediment trials	4.344	0.037
Wood nymphs vs. vegetation nymphs in wood-vegetation trials	13.303	0.000
Sand nymphs vs. dark sediment nymphs in sand-dark sediment trials	1.978	0.160
Sand nymphs vs. vegetation nymphs in sand-vegetation trials	9.899	0.002
Dark Sediment nymphs vs. vegetation nymphs in dark sediment-vegetation trials	7.200	0.007

In the trials using nymphs collected from wood substrates, we found nymphs on their preferred substrate more frequently than on any other substrate. The probabilities of nymphs collected from wood randomly selecting their preferred substrate over sand, vegetation or dark sediment were low (Table 1;  $p < 0.05$  for all). Because all of the calculated p-values were below



our chosen significance level, the observed pattern of habitat selection is not caused by random choice. We could not calculate p-values for the sets of trials for nymphs collected from dark sediment, vegetation or sand substrates because not enough data points were collected to use a normal approximation (Tables 1a, 2a and 3a).

In the wood control trials, there was no evidence that nymphs collected from wood substrates chose a substrate based on direction in our experiments (Table 1;  $p > 0.05$ ). In our dark sediment, vegetation and sand control trials, a binomial model was utilized and probabilities were produced that approach the upper-limit probability, indicating that there was little evidence of directional bias (Tables 1a, 2a and 3a).

In the comparison of substrate choice between nymphs collected from wood and nymphs collected from sand, we found a statistically significant difference in substrate preference ( $X^2$ : 8.438,  $p < 0.05$ , Table 2). Nymphs collected from wood were more likely to choose a wood substrate over a sand substrate, while nymphs collected from sand were more likely to choose sand over wood (Figure 2). The same was true when comparing substrate choice of nymphs found on wood with nymphs found on dark sediment ( $X^2$ : 4.344,  $p < 0.05$ , Table 2) and nymphs found on with nymphs found on vegetation ( $X^2$ : 13.303,  $p < 0.05$ , Table 2). In both cases, nymphs from wood were more likely to choose a wood substrate over vegetation or dark sediment, and nymphs found on vegetation or dark sediment were more likely to choose their respective preferred substrates (Figure 3 and 4).

Comparing the substrate choice of nymphs collected from sand with nymphs collected from dark sediment, no significant difference was found between the two populations ( $X^2$ : 1.978,  $p > 0.05$ , Table 2) This suggests that nymphs collected from sand were not more likely to choose

their preferred substrate than dark sediment, nor were nymphs collected from dark sediment more likely to choose sediment over sand (Figure 5).

When comparing substrate choice of nymphs found on sand with nymphs found on vegetation, we found that there was a significant difference between the two populations ( $X^2$ : 9.899,  $p < 0.05$ , Table 2). These data would imply that nymphs from sand were more likely to choose sand as a substrate than vegetation, and nymphs from vegetation were also more likely to choose their preferred substrate (Figure 6). The same results were found between nymphs collected from dark sediment and from vegetation ( $X^2$ : 7.200,  $p < 0.05$ , Table 2). Both nymphs collected from dark sediment and from vegetation were more likely to choose their preferred substrate than another substrate.

## **Discussion**

The results generated from Pearson's Chi-Square tests and binomial-normal model were statistically significant and supported our hypothesis for nearly all treatment combinations. As predicted, nymphs were predisposed to return to their preferred substrate, indicating that decision-making for substrate type and location is not completely determined by cryptic strategy (Figures 2, 3, 4, 5, 6 and 7). Therefore, our hypothesis was supported for all combinations of tanks, with the exception of nymphs from sand with wood or dark sediment.

In all trials run with nymphs collected from wood, we found that the nymphs chose their preferred substrate more often than any other substrate, supporting our hypothesis (Tables 1 and 2; Figures 2, 3 and 4). Furthermore, although we were unable to generate p-values for trials involving nymphs from vegetation or dark sediment, our binomial and  $X^2$ -values both support

our hypothesis (Tables 1a, 2 and 2a; Figures 3, 4, 5, 6 and 7). These findings may be explained by the fact that instantaneous anti-predator decisions are necessary to survive in aquatic habitats. Past research suggests that these survival-based decisions are strongly determined by an organism's familiarity with a given substrate (Glaser and Goldsteinholm, 2012).

López (2000) found that more successful and decisive substrate choices during a predator attack on Amphisbaenians (legless lizards) occurred more frequently when they were able to return to familiar substrate. The tendency of the Odonate nymphs to occupy a familiar substrate may be attributed to selection for nymphs that revert to what they know to be safe in the past—even when an unfamiliar substrate may be more cryptic. Anti-predator adaptations allow an organism to decrease hesitation time in the presence of a predator, leading to higher escape rates (Glaser and Goldsteinholm, 2012). A separate study examining rainbowfish determined that individuals who were familiar with their environment exhibited more efficient anti-predator responses and were more able to effectively recognize risks and escape danger (Brown, 2001). This reinforces the results of our Pearson's Chi-Square tests (Table 2), as nymphs similarly chose what was preferred and familiar over what was most cryptic.

In all trials run with nymphs from plants, individuals were significantly more likely to choose their preferred substrate over any other substrate (Table 2; Figures 3, 6 and 7). Moreover, during experimental trials, the most search time was required to locate nymphs when they were hidden within the vegetative substrates. The idea that vegetation is a common substrate inhabited by Odonate nymphs is relatively well-known. For example, in 2009, Remsburg and Turner found a positive correlation between wetland plants and density of *Gomphidae* nymphs in a sample of northern U.S. lakes. Besides serving as a cryptic substrate for anti-predator defense, Odonate

nymphs may be more successful on substrates that allow them to efficiently emerge from the water when they are ready to complete their terminal molt (Remsburg and Turner, 2009). This idea directly supports our findings, as the aquatic vegetation from which we collected nymphs either stuck out of the water or was found in close proximity to the shoreline.

Likewise, almost all the nymphs we collected from wood substrates were found on logs and branches that were at least partially out of the water. Remsburg and Turner (2009) found that substrates that extend out of the water decrease Odonate nymphs' time exposed to aquatic predators, therefore increasing their fitness. It is therefore reasonable to conclude that wood and vegetation are the most preferred substrates for crypsis and future fitness in general.

Selective pressures differ greatly for nymphs living on wood substrates versus substrates other than wood, which could explain why the number of nymphs collected from wood was much higher than the numbers collected from the other three substrates. Less cryptic substrates such as sand confer a selective disadvantage to nymphs, causing them to be less likely to reach adulthood and leave offspring (Folsom and Collins, 1984). When comparing binomial values within the four treatments, the values are highest when nymphs from sand and dark sediment have the opportunity to hide on a wood substrate (Tables 1a and 3a). This indicates that cryptic strategy is being simultaneously considered along with familiarity, and could explain the insignificant results for nymphs on sand (Tables 2 and 3a ; Figures 2 and 5). This may illustrate that sand is a less cryptic substrate, providing evidence as to why many of the nymphs from sand chose more cryptic substrates over their preferred one.

Although our hypothesis was supported by past research and our data from trials on nymphs taken from wood, we did not find significant differences in substrate choice throughout

the sand treatments (Figure 8). While nymphs from sand were more likely to choose sand as a substrate over vegetation (Tables 2 and 3a; Figure 6), no significant difference was found between sand and dark sediment or wood (Tables 2 and 3a; Figures 2 and 5). Discrepancies in the results for sand nymphs with wood and dark sediment may be attributed to the fact that wood and dark sediment are much more cryptic than sand, therefore outweighing the influence of familiarity.

There are some noteworthy sources of error to consider when analyzing the results of this study. There could have been inaccuracies in determining which nymphs were actually collected from their preferred substrates and which nymphs had been relocated by water disturbance or moved to a new location of their own accord. Although Moum and Baker (1998) suggested that Odonate nymphs do not tend to stray far from their natal substrate, when dragonflies are ready to begin their terminal molt during the late spring or summer, they abandon their aquatic habitat and seek the shoreline for drier conditions (Stoks and Córdoba-aguilar, 2011). Our study was conducted during the molting season, therefore we cannot be certain whether we found some of the nymphs on their preferred substrate, or a substrate that was used temporarily in transit to the shore. Over the course of our trials, fourteen of the nymphs we collected completed their terminal molts and had to be replaced.

In order to give our results greater significance and natural context, there are a number of aspects within this experiment that could be altered or improved for future studies of Odonate nymphs. Our results were partially limited by the fact that we were only able to calculate p-values for nymphs collected from one of the four substrates. Including more trials for all four substrates would make our results more conclusive. Additionally, studying the daily movements

and activities of the nymphs before experimental trials could reduce uncertainty in our assumptions about preferred substrate. Using transects on the shore of Douglas Lake and mark-and-recapture methods could potentially provide more insight into the movement patterns of Odonate nymphs, thus preventing mistakes in future collection.

It would also be useful to look at certain aspects of the nymphs collected in more detail. There was a great deal of variation in color and morphology of the nymphs collected, therefore there may be much more to learn through the quantification of pigment composition and behavioral patterns in relation to substrates. With a pigment analysis, we would be able to compare the pigments of individual nymphs to see how closely they resembled the background color of their preferred substrate. A behavioral study would give us more information as to how individuals taken from a preferred substrate behave on a non-preferred substrate. From there it could also be beneficial to introduce a predator in both situations, followed by an assessment to examine any behavioral differences based on predator presence. With a better understanding of how Odonate nymphs are able to conceal themselves, along with how they behave in different environments, choices based on familiarity, preference and crypsis could be better understood and applied.

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## Appendix

Table 1a. Binomial analysis of nymphs collected from a dark sediment substrate

Conditions of Aquarium	Binomial Value	Upper-limit Binomial Value	Lower-limit Binomial Value
Dark Sediment and Dark Sediment (Control)	0.205	0.246	0.001
Dark Sediment and Sand	0.044	0.246	0.001
Dark Sediment and Vegetation	0.044	0.246	0.001
Dark Sediment and Wood	0.117	0.246	0.001

Table 2a. Binomial analysis of nymphs collected from a vegetation substrate

Conditions of Aquarium	Binomial Value	Upper-limit Binomial Value	Lower-limit Binomial Value
Vegetation and Vegetation (Control)	0.205	0.246	0.001
Vegetation and Dark Sediment	0.044	0.246	0.001
Vegetation and Sand	0.044	0.246	0.001
Vegetation and Wood	0.010	0.246	0.001

Table 3a. Binomial analysis of nymphs collected from a sand substrate

Conditions of Aquarium	Binomial Value	Upper-limit Binomial Value	Lower-limit Binomial Value
Sand and Sand (Control)	0.246	0.246	0.001
Sand and Dark Sediment	0.246	0.246	0.001
Sand and Vegetation	0.010	0.246	0.001
Sand and Wood	0.246	0.246	0.001

Sand Treatment

Tank 1

Sand	Sand
------	------

Tank 2

Sand	Dark Sediment
------	---------------

Tank 3

Sand	Wood
------	------

Tank 4

Sand	Vegetation
------	------------

Wood Treatment

Tank 1

Wood	Wood
------	------

Tank 2

Wood	Sand
------	------

Tank 3

Wood	Vegetation
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Tank 4

Wood	Dark Sediment
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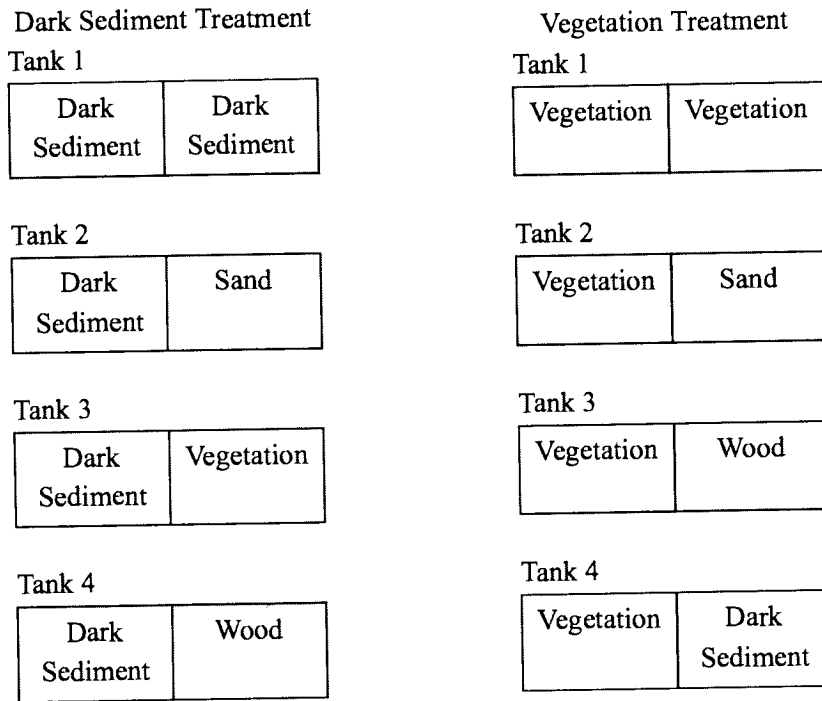


Figure 1. Diagrams of all control and experimental tanks used for testing substrate choices

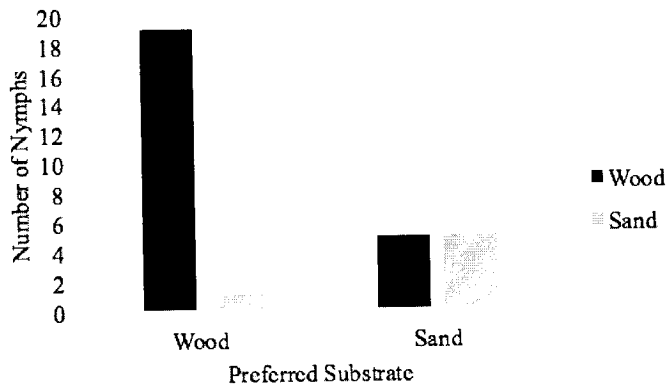


Figure 2. Substrate choice of nymphs collected from wood and substrate choice of nymphs collected from sand

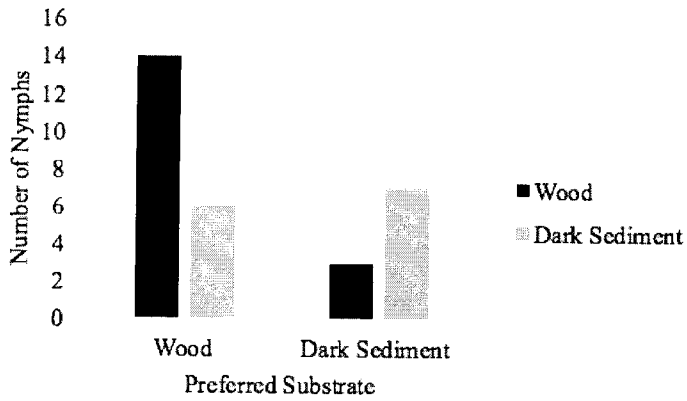


Figure 3. Substrate choice of nymphs collected from wood and substrate choice of nymphs collected from dark sediment

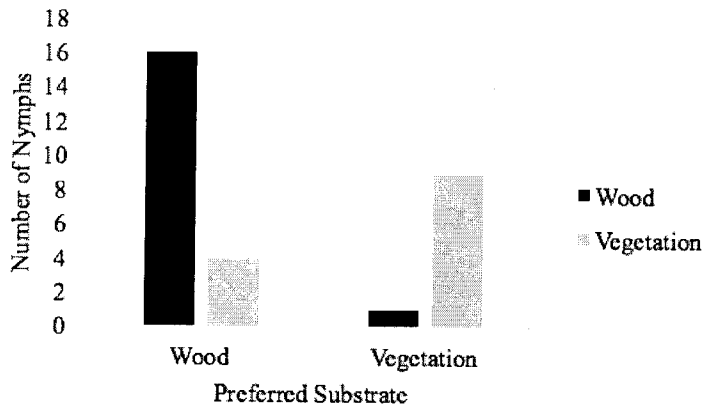


Figure 4. Substrate choice of nymphs collected from wood and substrate choice of nymphs collected from vegetation

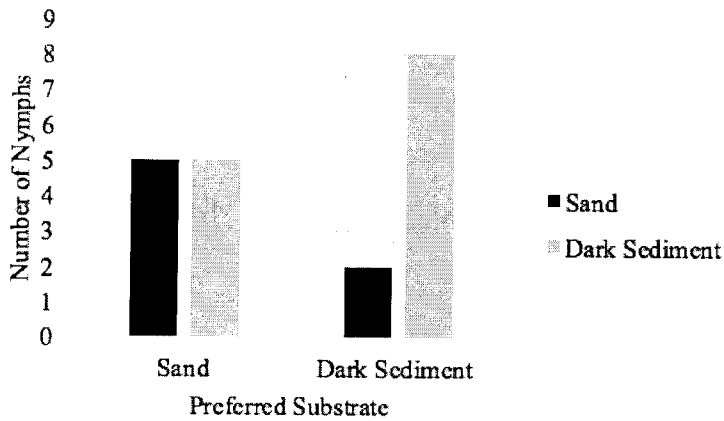


Figure 5. Substrate choice of nymphs collected from sand and substrate choice of nymphs collected from dark sediment

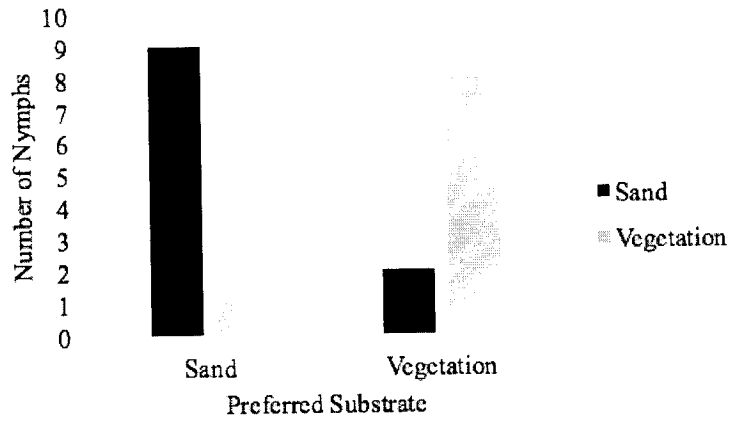


Figure 6. Substrate choice of nymphs collected from sand and substrate choice of nymphs collected from vegetation

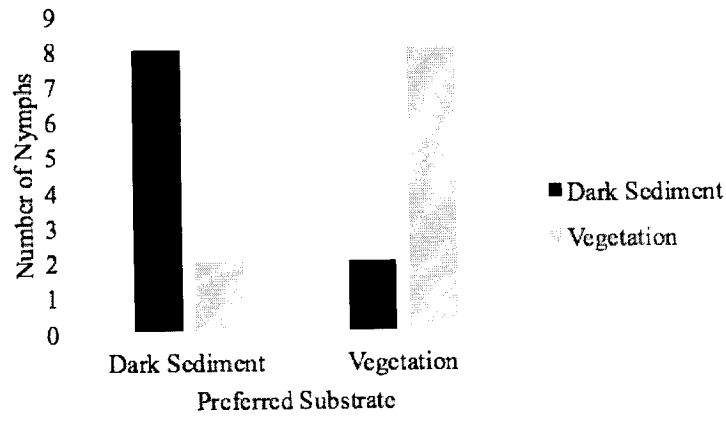


Figure 7. Substrate choice of nymphs collected from dark sediment and substrate choice of nymphs collected from vegetation

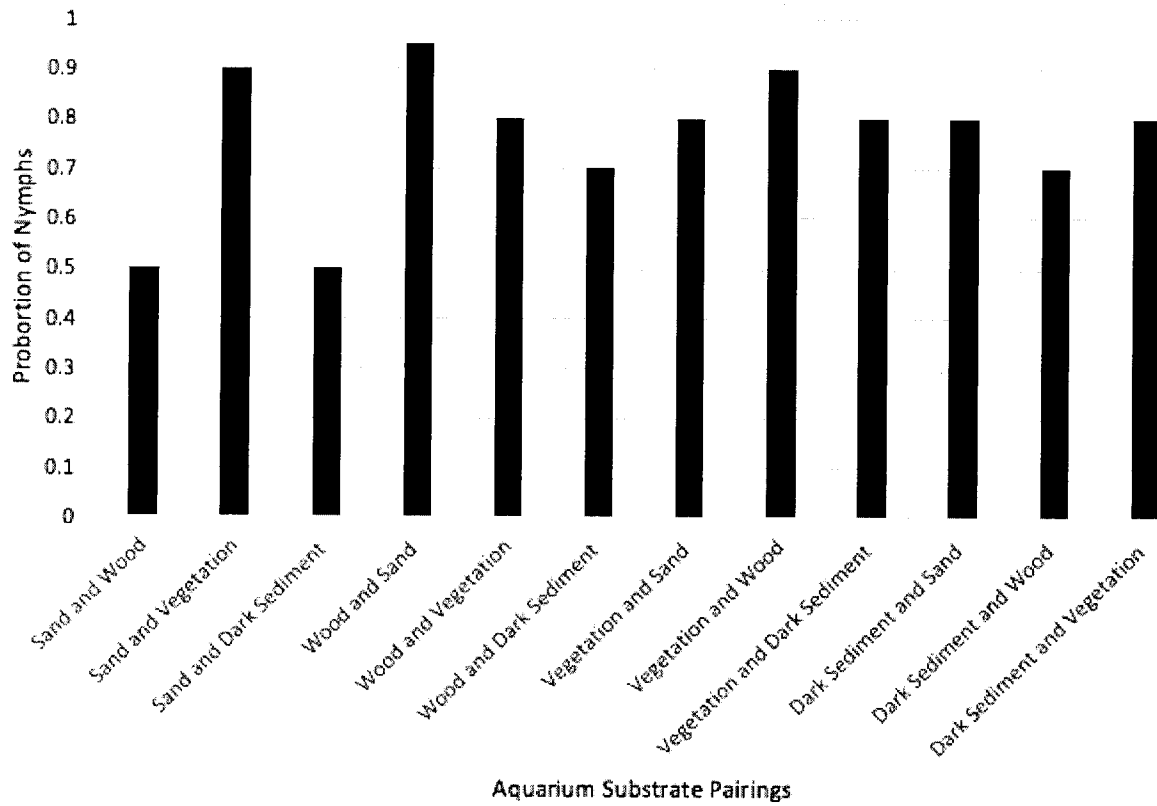


Figure 8. Proportions of nymphs choosing their preferred substrate in each aquarium substrate pairing