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OPTIMIZATION OF LABORATORY ASSAYS OF DIEL VERTICAL MIGRATION OF *Daphnia pulex* IN DETERMINING FISH KAIROMONES USING BACTERIAL DEGRADATION

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Approval of the Graduate School of Natural and Applied Sciences

Prof. Dr. Tayfur ÖZTÜRK
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Prof. Dr. Mesude İŞCAN
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Assist. Prof. Dr. Ayşegül OZAN
Co-Supervisor

Assoc. Prof. Dr. Meryem BEKLİOĞLU
Supervisor

Examining Committee Members

Prof. Dr. Nilgün KAZANCI

Prof. Dr. Feride SEVERCAN

Prof. Dr. Mesude İşcan

Assist. Prof. Dr. Ayşegül OZAN

Assoc. Prof. Dr. Meryem BEKLİOĞLU

ABSTRACT

OPTIMIZATION OF LABORATORY ASSAYS OF DIEL VERTICAL MIGRATION OF *Daphnia pulex* IN DETERMINING FISH KAIROMONES USING BACTERIAL DEGRADATION

Zorlu, Pelin

M.Sc., Department of Biological Sciences

Supervisor: Assoc. Prof. Dr. Meryem Beklioğlu

Co-Supervisor: Assist. Prof. Dr. Ayşegül Ozan

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Diel Vertical Migration (DVM) is a widespread predator-avoidance strategy in *Daphnia* during which as a normal pattern they descent to hypolimnion during day and ascent to epilimnion with dusk. This response has been shown to be induced by chemical cues (kairomone) exuded by predator fish. In this study, which is a part of an interdisciplinary study, it was aimed to investigate the impact of bacterial biodegradation of the fish kairomone using DVM response of *D.pulex* originated from Lake Eymir and cultured in the laboratory. To meet such goal, several optimization experiments which included DVM performance of different species of *Daphnia*, different clones of *D.pulex* and the varying food levels were carried our to explore the best experimental design for determining the impact of bacterial degradation.

In optimization experiments, comparison of the DVM responses of the *Daphnia pulex* and *Daphnia magna* revealed no significance. Different *Daphnia pulex* clones (migrating vs. nonmigrating) both performed DVM but the difference was rather related to the amplitude of migration. In the food optimization experiment, stronger migrations were recorded when food is abundant in response to fish kairomone. Furthermore, there was no coupling in responses to morphological features and DVM of *Daphnia* in the fish cue and varying food level treatments. Therefore, the results of this study recommended following experimental conditions for future studies: *D.pulex* indivuals collected from hypolimnion and fed with 1 mg C l^{-1} fresh algal culture appeared to provide an optimum DVM experimental condition. In DVM experiments with varying bacterial density, the test individuals in fish cue (F) treatment performed DVM whereas the test individuals in control treatments stayed above the thermocline in all experiments. However, the responses to the varying bacteria density treatments were not conclusive to show the impact of varying bacterial density on the kairomone activity since both the degradation and the lack of degradation responses were recorded in response to the bacterial enrichment.

In spite, these results show that the experiments carried out in this study achieved to show the impact of fish kairomones on DVM response of *D.pulex*. It can be concluded that this study was successful to simulate the effect of fish predation in the laboratory environment.

Key words: *Daphnia pulex*, kairomone, diel vertical migration, bacterial biodegradation, fish cue.

ÖZ

BALIK SİNYALLERİNİN BAKTERİYEL BİYOYIKIM KULLANILARAK TANIMLANMASI AMACIYLA *Daphnia pulex*'İN GÜNLÜK DİKEY GÖÇ LABORATUVAR DENEYLERİNİN OPTİMİZASYONU

Zorlu, Pelin
Yüksek Lisans, Biyolojik Bilimler Bölümü
Tez danışmanı: Doç. Dr. Meryem Beklioğlu
Ortak Tez Danışmanı: Yard. Doç. Dr. Ayşegül Ozan
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Günlük Dikey Göç (GDG) *Daphnia*'nın gündüz hipolimniyona inmek, gece ise ters yönde epilimniyona doğru çıkmak suretiyle gerçekleştirdiği çok yaygın bir avlanma önleme taktiğidir. Bu davranışa avcı balıktan salgılandığı düşünülen kimyasal sinyallerin (kairomone) neden olduğu gösterilmiştir. Disiplinlerarası bir projenin bir kısmı olan bu çalışmada, Eymir Gölünden getirilmiş ve laboratuarda yetiştirilmiş *D.pulex* 'in GDG davranışı vasıtasıyla bakteriyel biyoyıkımın balık sinyali üzerindeki etkisinin incelenmesi amaçlanmıştır. Bu amaç doğrultusunda, bakteriyel biyoyıkımın etkisini belirlemede en iyi deney koşullarını yaratmak için, farklı *Daphnia* türlerinin, farklı *D.pulex* klonlarının ve değişik besin konsantrasyonlarının GDG performanslarının karşılaştırmasını da içeren çeşitli optimizasyon deneyleri gerçekleştirilmiştir. Sonuç olarak, *D.pulex* ve *D.magna* türlerinin GDG' lerinin karşılaştırması belirgin bir farklılık göstermemiştir. Farklı *D.pulex* klonlarının ikisi de (göç eden ve göç etmeyen) GDG davranışı

göstermiştir. Aralarındaki farkın daha çok göçün yoğunluğu ile ilgili olduğu gözlenmiştir. Besin deneyinde, balık sinyalinin varlığında, besin konsantrasyonunun çok olduğu işlemlerde daha güçlü göç davranışı gözlenmiştir. Ayrıca, morfolojik değişimler ile balık sinyali ve değişik besin konsantrasyonlarında gözlenen GDG davranışı arasında bir bağlantı bulunmamıştır. Buradan yola çıkarak, bu çalışmanın sonuçları gelecek çalışmalarda gözönünde bulundurulmak üzere aşağıdaki deney koşullarını önermektedir: hipolimniyondan toplanan ve 1 mg C l^{-1} alg kültürüyle beslenen *D.pulex* bireylerinin kullanılmasının en uygun GDG deney koşullarını sağladığı gözlenmiştir. Değişik bakteri yoğunluklu GDG deneylerinde, kontrol işlemindeki bireyler termoklinin üzerinde kalırken, balık sinyali içeren işlemdeki (F) bireyler göç etmişlerdir. Ancak, değişik bakteri yoğunluklu işlemlere verilen davranışsal tepkiler, aynı zamanda bakteri zenginleştirmesi sonucunda hem biyoyıkıma hem de biyoyıkım olmamasına işaret ettiği için değişik bakteri yoğunluklarının sinyalin aktivitesine etkisini göstermek açısından kesin bir sonuca götürmemektedir. Buna rağmen, bu çalışmada gerçekleştirilen deneyler balık sinyalinin *Daphnia*'nın GDG davranışı üzerindeki etkisini başarıyla göstermiştir. Sonuç olarak, bu çalışma balık avlanma baskısının etkisini laboratuar koşullarında başarıyla canlandırmıştır.

Anahtar kelimeler: *Daphnia pulex*, kairomone, günlük dikey göç, bakteriyel biyoyıkım, balık sinyali.

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INTRODUCTION

1.1. Chemical communication in aquatic environments

In recent years, there has been an increasing interest in understanding the importance of non-visual signals for communication in aquatic systems. Poor light transmission in the water, and high habitat complexity reduce the use of vision for communication in aquatic organisms. Therefore, chemical communication has been shown to be important in locating food in molluscs, crustaceans, echinoderms and fish (Poulet & Marsot, 1978; Carr, 1988) and partner in fish and brown algae (Muller et al., 1979; Sorensen, 1992; Olsen et al., 2000), in kin recognition in fish and tadpoles (Blaustein & Waldman, 1992; Olsen, 1992; Brown & Brown, 1996), as well as predation avoidance (reviewed by Kats & Dill, 1998). Many studies have shown that aquatic organisms from different taxa and functional groups respond to minute concentrations of chemical substances released by other organisms (see reviews Brönmark & Hansson, 2000; Burks & Lodge, 2002). One of the most well-studied responses to such a chemical is the response to chemical substance released by a predator fish and perceived by zooplankters. As a pioneering study, Dodson (1988a) experimentally showed that *Daphnia* responded to fish incubated water as if the actual predators were present, and therefore suggested that the cue was a chemical released by the predator.

Numerous field and laboratory studies have shown responses of prey organisms in response to predator cue (kairomone) in a diverse set of taxa for marine and freshwater snails (Snyder & Synder, 1971; Alexander & Covich, 1991), larval amphibians (Kats et al., 1988; Skelly & Werner, 1990; Laurila, 2000), many fish species (Hirvonen et al., 2000; Mathis et al., 2000; Pettersson et al., 2000), zooplankton (Lampert, 1993; Lauridsen et al., 1996; Burks et al., 2000), and some flagellated algal taxa (Hansson, 1996, 2000). Moreover, many laboratory studies have shown anti-predator defence in response to chemical cue released by the predator and injured conspecifics, which act as alarm cue, in tadpoles of wood frog and American toad (Petranka & Hayes, 1998), in salmonids (Mirza & Chivers, 2001), and amphipod crustaceans such as *Gammarus* (Mathis & Hoback, 1997; Wudkevich et al., 1997).

1.2. Nature of the kairomones

Most compounds, which are known as info-chemicals, kairomones, or cues that have been suggested to act as cues have been neither isolated, nor purified, or structurally identified through complete biochemical assays though a limited number of physical and chemical properties has been characterized so far.

Von Elert & Franck (1999) characterized the active chemical cue released by *Daphnia*, which induced colony formation in green algae *Scenedesmus* as an olefinic, low-molecular-weight (<500 Dalton) carboxylic acid. Concentration of 1 liter of *Daphnia*-cued water using solid-phase extraction produced similar colony formation in *Scenedesmus* (Lürling & Von Elert, 2001). Collectively, these

studies suggested that the daphnid kairomone responsible for colony formation was moderately lipophilic.

Furthermore, using gas chromatography-mass spectroscopy methods Wendel & Jüttner (1997) identified measurable quantities of heptadecene-1 within swarms of a zooplankton species. They suggested that this might be the cue which maintains conspecifics within the swarm.

Many freshwater macrophytes release chemical cues for deterring herbivory (Lodge et al., 1998). Only a few of them have been identified or quantified. For example, Newman et al. (1996) demonstrated that glucosinolate deterred many herbivores from watercress (*Nasturtium officinale*). In addition, habenariol deters crayfish from feeding on the freshwater orchid, *Habenaria repens* (Bolser et al., 1998; Wilson et al., 1999), and numerous lignoids deter the same crayfish from feeding on another freshwater macrophyte, which is lizard's tail, *Saurus cernuus* (Kubanek et al., 2001). Besides defensive purposes, specific plant compounds affect competition between submerged macrophytes and phytoplankton. For example, *Myriophyllum spicatum* (Eurasian water milfoil) releases a hydrolysable polyphenol, tellimangradin II, which deters algal growth (Gross et al., 1996).

The chemical signals released from predator and perceived by prey organisms that induce different defense strategies, i.e. Diel Vertical Migration, Diel Horizontal Migration, morphological and life-history changes, in several zooplankton species are shown to be water-soluble substances exuded experimentally by different predators (Dodson, 1988a, Loose et al. 1993). Loose et al. (1993) carried out series of experiments to reveal several features of fish kairomones. They showed that the kairomone was readily released into the water by fish that after 60 min of

incubation of the fish, water already contained kairomone which induced DVM response in *Daphnia*. Further increase of fish incubation time did not change the positive behaviour. Although further bioassays showed that the treatments with faeces, mucus and hindgut of fish could provoke a significantly deeper mean day-depth than the control, none of these treatments induced a significant DVM in *Daphnia*. Dilution of these water with control water (1:10) resulted in a strong decrease of mean day-depth whereas fish water diluted in the same way was still highly active. Moreover, all of the tested fish species whether planktivorous or piscivorous provoked the DVM response in *Daphnia*. It was suggested that fish cue was not very specific, if not identical since all fish species have a either short or long juvenile stage at which are plankton predators. Additionally, the type of food given to the fish did not affect the release or the activity of the kairomone such as hungry fish produced the same results as well-fed ones. Kairomone has been also shown being a low-molecular-weight (<500 Dalton) and non-volatile compound that is stable to extreme temperatures within the range of –20° C to +120° C and pH conditions within the range of pH 0.8 to pH 14 (Parejko & Dodson, 1990; Loose et al., 1993). Moreover, its resistance against two different general peptidases, which are Pronase E and Proteinase K, it seems unlikely that the kairomones are protein (Loose et al., 1993). However, after incubation for 24 h at 37° C without prior sterile filtration, *Daphnia* did not perform DVM, whereas incubation at 4° C under the same conditions left the water active. Therefore, it was suggested that the kairomone was readily degraded by bacteria (Dodson 1988a; Loose et al. 1993). Quick release of the kairomone with the easy microbial degradation seems to serve as mechanisms for the high reliability of the signal

(Loose et al., 1993). Consequently, it is suggested that the chemical has a high turnover in the water (Larsson & Dodson, 1993; Loose et al., 1993). Furthermore, it is also suggested that the amplitude of DVM in zooplankton is a function of predator abundance in the environment. A tight response of the DVM behaviour to both increase and decrease of planktivorous fish was observed both in the field (Ringelberg et al., 1991) and in experiments (Bollens & Frost, 1991; Loose, 1993a) Additionally, Loose & Dawidowicz (1994) have shown experimentally that above a threshold kairomone concentration, the strength of migration increased with increases in the concentration of fish exudates. (see also von Elert & Pohnert, 2000).

Von Elert & Loose (1996) suggested a method to enrich the cue from fish incubated water by sorbent extraction. According to this method, the chemical nature of the kairomone released by three cyprinid species has been characterized as a nonolefinic, low-molecular weight compound of intermediate lipophilicity that hydroxy groups are essential for activity. Moreover, purification using HPLC yielded only a single active fraction with retention times identical for kairomones released from different species of fish (Von Elert & Pohnert, 1996). This result matched with the following HPLC purification results for three cyprinid species (Von Elert & Loose 1996). Therefore, there was no or a rather low predator specificity of the kairomones even across families indicating that the active compounds are very similar if not identical (Von Elert & Loose, 1996; Von Elert & Pohnert 2000). Von Elert & Pohnert (2000) hypothesized that the kairomones are adsorbed on to food particles due to being lipophilic and is sensed by *Daphnia* via ingested food items. However, their results clearly demonstrated that DVM-

inducing chemicals was not mediated via food particles. Therefore, the cue ought to be perceived as a freely dissolved molecule.

As the production of the kairomone seems to be disadvantageous to the predator, selection against the release of these cues might be expected. As kairomone production is widespread and of low species specifity in freshwater fish, Ringelberg & Van Gool (1998) argued two possible explanations which might hold for the existence of fish kairomones: (i) these kairomones are essential for the functioning of the predator and the negative effects are counterbalanced and (ii) the kairomones are not produced by the predator at all. In the latter case, it is assumed that some other organism closely associated with the predator is responsible for the production. To investigate the second hypothesis, Ringelberg & Van Gool (1998) treated a juvenile perch with ampicillin. In the presence of fish kairomone DVM of *Daphnia*, as compared with the control, was evident. On the other hand, this was reduced markedly when the fish was treated with ampicillin though the enhancing effect had not disappeared completely. Moreover, ampicillin was added afterwards to water taken from the aquarium with fish, and compared to untreated fish water that both treatments enhanced DVM. Furthermore, the inhibiting effect of ampicillin on kairomone production was reversible. A week later, fish water prepared with a previously ampicillin-treated fish enhanced DVM as intense as untreated fish water. Therefore, Ringelberg & Van Gool (1998) claimed that not fish, but bacteria associated with fish, probably present in the mucus cover of fish, are responsible for the production of kairomone. They suggested that the same kairomone –producing bacteria, or just possibly different bacteria producing the same kairomone, occur on different

kinds of freshwater fish. Based on these findings, Boriss et al. (1999) tested trimethylamine (TMA) as a possible candidate for the active factor. Bacteria in the skin of fish release TMA, giving the odour characteristic of rotten fish, is a main metabolite of fish-borne trimethylamine-N-oxide (TMAO), which is involved in osmoregulation of euryhaline and marine fish (Yancey & Somero, 1979; Van Waarde, 1988) Boriss et al. (1999) found that trimethylamine (TMA) induced vertical migration in *Daphnia*. However, TMA is a very common substance in nature released in many degradation processes (King, 1984) and used as substrate in methane formation (de Angelis & Lee, 1994) and even released by zooplankton (de Angelis & Lee, 1994). Following this study, Sakwinska (2000) showed that TMA did not trigger antipredatory life-history responses in *Daphnia*. Moreover, Pohnert & Von Elert (2000) showed that the efficient removal of existing TMA traces from fish incubation water did not decrease the kairomone activity. Therefore, it was concluded that TMA is most little likely to be the kairomones inducing anti predator changes in *Daphnia* (Pohnert & Von Elert, 2000; Sakwinska, 2000; Lass et al., 2001).

Furthermore, Forward & Rittschof (1999, 2000) suggested that disaccharide degradation product of fish external mucus containing sulfated and acetylated amines could serve as kairomone and enhance DVM behaviour in brine shrimp and crab larvae. They showed that the DVM response was enhanced as the concentration of fish mucus derived from a marine fish increased. Dissaccarides originating from polysaccarides such as chondroitin sulfate A and heparin also provoked DVM as compared to responses in filtered and aged seawater.

In contradiction with several studies which suggested the external fish mucus both in freshwater and marine fish as the source of kairomone (Ringelberg & Van Gool, 1998; Forward & Rittschof, 1999, 2000), Von Elert & Pohnert (2000) found that the kairomone was not released from mucus by digestion with hyaluronidase. It was also suggested previously that fish mucus did not induce DVM behaviour in *Daphnia* (Loose et al., 1993). Additionally, Von Elert & Pohnert (2000) argued the results of the study carried out by Ringelberg & Van Gool (1998), and emphasized that even after antibiotic treatment, the incubation water was still half as active as incubation water of non-treated fish. They further hypothesized that incubating a perch in 8 l of water for 48 h might lead to elevated bacterial metabolism and hence to an overestimation of the bacterial contribution to the release of the kairomone. However, the origin of the cue/cues for inducing vertical migration is still unknown.

As the increasing number of studies on the nature of the kairomones provides more detailed information, a number of advantages would result from knowing its chemical nature: it would open then possibility for controlled experiments. It would also allow predictions about the dynamics of the kairomone in the lake, which in turn could help explain various seasonal and species-specific patterns of DVM observed in the field. It would also allow comparisons between behavioural and morphological signals (Loose et al., 1993).

Once the structure is known, it will be possible to develop field assays for finding the concentration of the signal in the lake. This may provide a new method for rapid assessment of fish population and/or activity in a lake (Larsson & Dodson, 1993; Loose et al., 1993).

1.3. The role of *Daphnia*

In pelagic freshwater systems *Daphnia* is an important food source for fish and the abundance of these herbivorous crustaceans has major effects on water clarity, in turn on structure and dynamics of the whole food web (Lampert 1987a). Herbivorous zooplankton, especially *Daphnia* reduce algal biomass and change algal community structure (Elser & Goldman, 1990). Large-bodied *Daphnia*, in particular, graze a wider size-range of phytoplankton (Lampert, 1987b) than smaller zooplankton, but are more vulnerable to fish predation (Brooks & Dodson, 1965).

1.4. Predator avoidance strategies in *Daphnia*

Species of the genus *Daphnia*, one of the best-studied genera in freshwater environments, are very plastic in their responses to the chemicals exuded by their predators. They have been shown to exhibit predator-induced changes in morphology (i.e. helmet development, change in body size), in life history traits (i.e. size and age at maturity, size and number of eggs) and in behavioural traits (i.e. DHM, DVM). Each of these responses has been interpreted as an adaptive mechanism to avoid or counterbalance predation.

1.4.1. Fish kairomone-induced life history changes

Members of the genus, *Daphnia* have been shown to exhibit predator kairomones-induced life history changes. Many studies have shown experimentally life history changes such as reduction in maturation size and time, changes in egg and offspring size, clutch size, and production of sexual eggs, in response to fish

kairomones (Machacek, 1991; Stibor, 1992; Weider & Pijanowska, 1993; Slusarczyk, 1995; De Meester & Weider, 1999; Spaak et al., 2000). A number of studies on the environmental factors such as food level and temperature revealed synergistic effect with kairomone on life-history parameters of *Daphnia*. It was shown experimentally that the influence of the kairomone is important for making the animals more vulnerable to food level and vice versa (Hanazato et al., 2001; Slusarczyk, 2001; Weber, 2001). It was suggested that temperature and food level with fish kairomone might have strong effect on life history traits of *Daphnia* (Weetman & Atkinson, 2002), whereas Doksaeter & Vijverberg (2001) found no evidence for these three-way interactions.

1.4.2. Fish-induced morphological changes

Morphological adaptations including changes in size and shape are considered to allow *Daphnia* to reduce the risk of predation by size selective predators (Dodson, 1974; Krueger & Dodson, 1981; Dodson, 1989b; Tollrian, 1990; Boersma et al., 1998; Barry, 2000; Stibor & Navarra, 2000). Induced morphological changes result in prey either "too large" to be easily handled or swallowed or "too small" to be easily seen by visual predators, compared to the noninduced form. These changes are thought to be adaptive since they respond in a predator specific way in order to reduce the predation risk (Havel & Dodson, 1984; Parejko, 1990; Tollrian, 1995b). In laboratory experiments, it was shown that while in the presence of kairomone exuded by invertebrate predators, *Daphnia* developed neckteeth, elongated helmets (Krueger & Dodson, 1981; Dodson, 1988b; Hebert & Grewe, 1985; Dodson, 1989a), some of the observations of morphological

adaptations revealed longer tail spines, smaller body length in response to kairomone exuded by fish (Dodson, 1988b; Dodson, 1989a; Spaak & Boersma, 1997; Boersma et al., 1998). On the other hand, the morphological changes such as induction of helmets, neck teeth, and spines have been suggested to have costs (Parejko & Dodson, 1991). Therefore, animals, which develop such character, should show changes in life history traits such as reduction in reproduction, changes in maturation time as a result of these costs. However, several studies showed no strong association between the degree of neck teeth induction and life history characteristics (Spitze, 1992; Lüning, 1994; Tollrian, 1995a). It is suggested that induced responses in different traits (morphological, behavioural or life-history) can at least be partly uncoupled (Spitze, 1992; Lüning, 1994; De Meester & Pijanowska, 1996; Boersma et al., 1998).

1.4.3. Fish kairomone-induced behavioural changes

In shallow lakes, multiple studies documented heterogeneous horizontal distribution of cladocerans, especially *Daphnia* (Kairesalo, 1980; Timms & Moss, 1984; Visman et al., 1994; Lauridsen & Buenk, 1996; Moss et al., 1998). This daily horizontal displacement of zooplankton is called "Diel Horizontal Migration" (DHM) and characterized with a daytime horizontal migration towards the plant bed and nighttime reverse move to the open water. Predator-avoidance is experimentallt suggested to be the ultimate reason for daphnids to undergo DHM (Lauridsen et al., 1996). Additionally, the influence of macrophyte beds of the littoral on the horizontal distribution of zooplankton was suggested to depend on a trade-off between macrophyte avoidance and predation pressure (Lauridsen &

Lodge, 1996), as well as the nutrient state of the lake (Jeppesen et al., 1998). In a recent laboratory experiment, Burks et al. (2001) showed that daphnid mortality due to predation by fish declined with increasing macrophyte density. Moreover, in an earlier laboratory experiment, it was shown that *Daphnia* only increased their use of macrophyte in the presence of fish (Burks et al., 2000). In addition, experiments to investigate the impact of macrophyte cues on daphnid growth and reproduction revealed that macrophyte chemical cues suppressed growth and life-history traits resulting in delayed reproduction with fewer eggs.

Furthermore, various planktonic species both in marine and freshwater environments have been shown to perform a daily vertical displacement in the water columns with an amplitude which may vary from few meter to hundred of meters (Dodson, 1988a; Bollens & Frost, 1989; Dawidowicz et al., 1990; Neill, 1990; Tjossem, 1990; Ringelberg, 1991a; Dawidowicz, 1993; Loose, 1993a). This daily vertical displacement of plankton is called "Diel Vertical Migration", during which, as the normal pattern, zooplankton descend with dawn to dark, cold, less oxygenated, and nutrient poor hypolimnion and ascent with dusk to surface water to graze on algae.

1.5. Diel Vertical Migration

DVM behaviour of zooplankton is widely accepted as an example of behavioral antipredator defense (Zaret & Suffern, 1976; Stich & Lampert, 1981; Gliwicz, 1986; Lampert, 1989, 1993): zooplankton migrate to greater depths during the day to reduce their chance of being detected by visual predator fish. This hypothesis is strongly supported by many studies that DVM in many zooplankton species can

be induced by kairomones exuded by predators (Dodson, 1988a; Dawidowicz et al., 1990; Neill, 1990; Loose, 1993a; Pijanowska, 1993; Ringelberg at al., 1997; Beklioğlu & Jeppesen, 1999; Matthew et al., 1999). On the other hand, several environmental factors, known as proxy factors, have been suggested to influence the amplitude and the pattern of the migration along with fish kairomone such as food abundance in laboratory and field studies (Johnsen & Jakobsen, 1987; Pijanowska & Dawidowicz, 1987; Dini & Carpenter, 1992; Loose & Dawidowicz, 1994; Spaak & Boersma, 2001; Muluk & Beklioglu, submitted), level of predation pressure (Bollens & Frost, 1991; Loose, 1993a; Loose & Dawidowicz, 1994; Ringelberg et al., 1997; Pohnert & Von Elert, 2000), water temperature (Haney, 1993), concentration of dissolved oxygen (Wright & Shapiro, 1990; Gaso et al., 1995; Beklioglu & Jeppesen, 1999; Lass et al., 2000), and light intensity (Ringelberg, 1991b; Ringelberg & Flik, 1994; Ringelberg, 1999). Several hypotheses have been suggested on the influence of food availability on the DVM behaviour of zooplankton. Migration occurs only when food is abundant; only well-fed organisms can afford the cost of migration (Dagg, 1985; Johnsen & Jakobsen, 1987). On the other hand others suggested that migration is more likely when food is limiting (Giguere & Dill, 1980; Hoenicke & Goldman, 1987; Dini & Carpenter, 1992). A third explanation on migration centures around a vertical heterogeneity in food availability (Pijanowska & Dawidowicz, 1987). Several laboratory studies revealed that the animals in low food treatments stayed higher in the columns in response to fish cue (Loose & Dawidowicz, 1994; Spaak & Boersma, 2001), whereas Dini & Carpenter (1992) in a mesocosm experiment recorded stronger migration in lower food level.

Furthermore, a tightly coupled response of zooplankton towards increasing and decreasing abundance of fish has been observed experimentally. Loose & Dawidowicz (1994) showed that the strength of migration increased with increases in the concentration of fish exudates (see also Bollens & Frost, 1991; Loose, 1993a; Lass et al., 2000). On the other hand, vertical displacement is costly since individuals have to move to unfavourable conditions. In general, the cost is expressed as a reduction of growth and reproduction in laboratory experiments (Dawidowicz & Loose, 1992; Loose & Dawidowicz, 1994), as well as in field studies (Orcutt & Porter, 1983; Stich & Lampert, 1984; Kerfoot, 1985).

Furthermore, results of few studies indicated that induced responses of morphological and life-history traits could be at least partly uncoupled, expressed independent from each other (Spitze, 1992; Lüning, 1994; De Meester & Pijanowska, 1996). Boersma et al. (1998) investigated the responses of a number of morphological, life-history and behavioural (phototaxis and escape reaction) in *Daphnia magna* clones to the presence of fish kairomones. All clones reacted to the presence of fish kairomone with at least one trait, but none reacted with a shift in all of the traits studied. Therefore, it is suggested that the responses of different traits to the presence of fish kairomones are to a large extent uncoupled.

1.6. Phenotypic plasticity and genetic variation in *Daphnia*

Phenotypic plasticity can be defined as environmentally induced variation that may lead to the expression of different phenotypes for a given genotype. Many of these adaptive changes in phenotypes are a direct response to the presence of predators under given environmental conditions (Parejko & Dodson, 1991; De

Meester, 1996). For example, among different *Daphnia* species, the higher mean size, which is more vulnerable to size-selective predation by fish, showed a stronger reaction to fish kairomones than the smaller taxa, whereas the intrinsic rate of increase in small size taxa showed a stronger reaction (Spaak et al., 2000). Furthermore, genotypes may differ in their daytime depth preferences, as it has been shown by field studies using electrophoretic markers (Weider 1984, 1985; Müller & Seitz, 1993) and by in situ enclosure experiments (Leibold et al., 1994). De Meester & Weider (1999) suggested that there might be a coadaptation between day-depth selection and size-related traits in *Daphnia*, and smaller animals remaining in shallower water during the day, when compared with larger animals (see also De Meester, 1994; Reede & Ringelberg, 1995, 1998). Moreover, in an earlier study by De Meester et al. (1995) showed that the relationship between body size and depth selection behaviour not only exists between genotype (large-bodied vs. small-bodied clone) level (see also Leibold & Tessier, 1991; De Meester, 1994; Reede & Ringelberg, 1995, 1998), and also within-genotype (large vs. small adults of a given clone) level. Boersma et al. (1998) have suggested that the major distinction is not between inducible and noninducible genotypes but rather that the genotypes differ in the combination of traits such as morphological, life history or behavioural for which they show inducible responses, therefore the reactions of different traits are to a large extent uncoupled.

1.7. The scope of the study

This study is a part of an inter-disciplinary study including microbiology, biophysics and behavioural ecology funded by TÜBİTAK YDABÇAG (100Y035) was to explore nature of info-chemicals released by fish and sensed by *Daphnia.*

In this part of the interdisciplinary study, it was aimed to investigate the impact of bacterial biodegradation of the fish kairomone using DVM response of *D.pulex* originated from Lake Eymir and cultured in the laboratory. To meet such goal, firstly several experiments which included DVM performance of different species of *Daphnia*, different clones of *D.pulex* and the varying food levels were carried our to explore the best experimental design for determining the impact of bacterial degradation.

CHAPTER 2

MATERIAL AND METHODS

2.1. The experimental design

Different experimental setups have been designed depending on the focus of the study in order to observe the fish kairomone induced-DVM of *Daphnia* in laboratory conditions. Some researchers used Perspex tubes (approx. 1 m length, 1.5 cm diameter, closed at the bottom or flow-through) placed inside a thermally stratified waterbath illuminated from the top through frosted glass screens (Dawidowicz & Loose, 1992; Loose et al., 1993; Dawidowicz, 1993; Loose & Dawidowicz, 1994; Von Elert & Loose, 1996; Boriss et al., 1999; Lass et al., 2000; Von Elert & Pohnert, 2000). Furthermore, large indoor plankton towers (11.2 m high, 0.86 m in diameter, stainless steel, twin-tank systems) also have been extensively used in many studies (Lampert & Loose, 1992; Loose, 1993b; De Meester et al., 1995; Spaak & Boersma, 1997; Spaak & Boersma, 2001). On the other hand, completely different bioassays were used to test for the presence of fish kairomones, with the phototactic downward swimming response, caused by increases in light intensity. The apparatus consisted of a series of cylinders (7 cm in diameter, 100 cm in length), placed in a water bath (20°C) (Ringelberg & Van Gool, 1998) or two glass cylinders (40 cm high, 7 cm diameter) enclosed in

glass jackets containing water, and illuminated from above (Ringelberg & Van Gool, 1995)

The experimental setup used in this study was similar to the one used by Loose et al. (1993) and Dawidowicz & Loose (1992) to determine the DVM behaviour of individual zooplanktons in the laboratory.

The setup consisted of glass tubes (1m length, 1.5 cm diameters) placed into a thermally stratified water bath (110x90x15 cm) with 22°C water temperature at the top and 8°C water temperature at the bottom (Fig.1) to mimic thermal stratification in a deep lake (Fig.2). The water bath was transparent and illuminated from the top through frosted glass screens by two 220 V, 50 Watt halogen lamps to provide homogeneous and diffuse irradiation. The day and night cycle was kept at 16 and 8 h in a temperature-controlled climate room at 21±1°C room temperature, where the DVM experiments were carried out.

Each tube was filled with appropriate treatment water. Then, five 4-5 day old daphnids were placed into each tube in the evening. Throughout the experiment, every evening the food level was re-adjusted to the initial concentration by adding an appropriate amount of fresh concentrated algae from the top of the tubes. During the experiment, the depth of the individuals was recorded at 2-h intervals

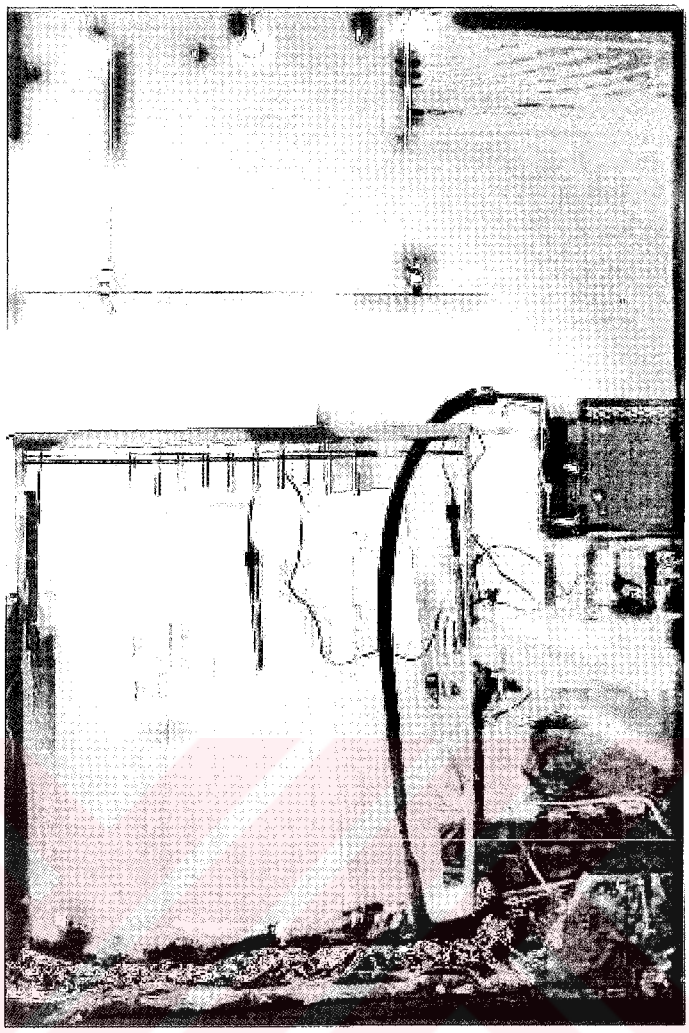

Figure 1. View of the DVM experimental setting in the climate room with 21°C average temperature kept under 16:8 day-night cycle; the water bath is thermally stratified with 22°C at the top and 9°C at the bottom.

five times during the day. The mean day-depths above 30 cm (animals in the warm epilimnion) indicated negative response; depths below 60 cm (animals in the cold hypolimnion) indicated positive response, which means existence of the diel vertical migration. (Dawidowicz & Loose, 1992; Loose et al., 1993, von Elert & Loose, 1996, von Elert & Pohnert, 2000). If depths occupied by daphnids were between 30-60 cm, it was considered as ambiguous and consequently neglected.

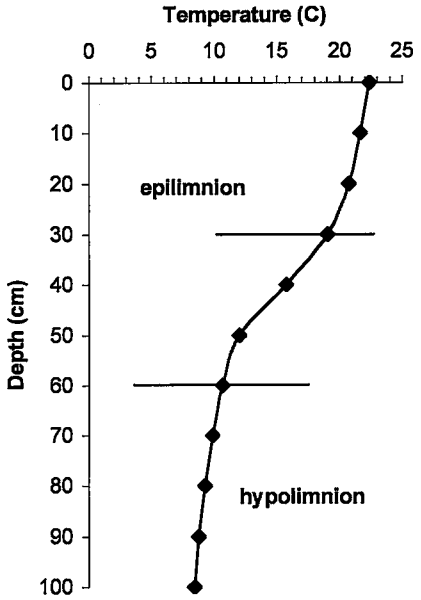

Figure 2. The temperature distribution in the test tubes (± C.I. 95%, 1 m length, 1.5 cm diameter).

2.2. Test organisms

Test animals were *Daphnia pulex* De Geer originating from Lake Eymir (Ankara, Turkey) where they coexisted with fish and invertebrate predators (Beklioğlu et al., in press; Beklioglu & Tan, submitted). The animals brought from the lake were cultured in the climate room to the sixth generation to avoid the maternal effect. Water in the aquariums in which *D.pulex* reared was brought from Lake Eymir. Prior to use, the water was filtered through 30 μm pore-sized plankton net to remove other zooplanktons and air-bubbled for ten days. The water in the aquariums was replaced once a week with this water which was filtered, aged and air-bubbled. Following the reproduction, neonates were separated using a Pasteur pipette regarding their generation. *Daphnia* were fed by a continuous chemostat-grown *Scenedesmus obliquus* green algae culture. The pure culture of *S.obliquus* was obtained from Göttingen University. The *S.obliquus* cultures were propagated

both in liquid and solid media using protease-peptone medium. The liquid protease-peptone medium was prepared as follows: 10 ml of each K_2HPO_4, $MgSO_4.7H_2O$, NaCl, $NaNO_3$, $CaCl_2.2H_2O$, KH_2PO_4, 1g protease-peptone, for 1 l of medium (see Appendix 1 for stock solutions). The solid medium was prepared following the same procedure with the addition of 15 g of agar for 1 liter of fresh algae medium. The culture was renewed every twenty days. Inoculations were realized in sterile laminar flow hood to prevent any contamination. Both solid and liquid cultures were kept in the climate room under constant temperature and light conditions. A magnetic stirrer was used to prevent algal cells to adhere to the bottom of the bottle.

Fish used in experiments to prepare fish water were sampled in Lake Eymir. Bleak (*Alburnus alburnus*) and tench (*Tinca tinca*), which were two of the most important predators on *D.pulex* in the lake were used. They were kept in lake water in the aquariums in the climate room. They were fed with live *Daphnia pulex* and dried fish food.

2.3. Preparation of the treatments

Control (C)

For the no-fish control (C), water taken from Lake Eymir was filtered through 0.45-μm cellulose-nitrate membrane filters (47 mm in diameter) to remove all particles. The filtered water was stored in 10 l pre-sterilized glass bottle and continuously sterile air-bubbled for two weeks prior to use.

Fish cue (F)

Two adult bleaks or tench (body length 5-6 cm) were transferred into 10 liter of the control water, where they were incubated for 24 h (Loose et al., 1993; Loose & Dawidowicz, 1994). Prior to use, the fish cue (F) was again filtered through 0.45-µm membrane filter.

Filtered fish cue (FF)

In the light of the previous study (Loose et al., 1993) suggesting that the kairomone can be degraded by bacteria, to investigate the role of the density of bacteria in the fish cue, three different treatments such as filtered fish cue (FF), incubated fish cue (IF) and incubated-filtered fish cue (IFF) were prepared. For FF, an appropriate amount of the fish cue (F) was filtered through 0.2-µm membran filter (47 mm in diameter) to remove bacteria. For the preparation of incubated fish cue (IF), an appropriate amount of fish cue (F) was incubated in the orbital shaker (G24 Environmental Incubator Shaker, New Brunswick Scientific Co. Inc.) at 180 rpm, at 37°C to provide a boost of bacterial growth. Two different incubation periods as 8 and 24 h were employed.

For the preparation of incubated-filtered fish cue (IFF), an appropriate amount of the IF was filtered through 0.2-µm cellulose nitrate membrane filter (47 mm in diameter) for the removal of bacteria.

2.4. The details of the experiments

Two different sets of experiments were carried out that included calibration of DVM experiments (i.e. DVM experiments with different *Daphnia* species and clones, and food levels), and the effect of varying bacterial density.

2.4.1. Calibration of DVM experiments using different *Daphnia* species and populations

This experiment was designed to compare the efficiency of DVM performed by *Daphnia pulex* versus *Daphnia magna* in the presence of fish cue. The experiment consisted of three treatments such as control *D.pulex* (C_{pulex}), fish cue *D.pulex* (F_{pulex}) and fish cue *D.magna* (F_{magna}). Each treatment was run in triplicates each of which contained three test individuals. *S.obliquus* was provided as fresh food with a concentration of 2.0 mg C L^{-1}.

Additionally, an experiment was designed to compare the DVM performance by *D.pulex* individuals collected from the upper part of the water column, epilimnion and the individuals collected from the bottom, hypolimnion. The experiment consisted of four treatments that included control-epilimnetic *D.pulex*, fish-epilimnetic *D.pulex*, control-hypolimnetic *D.pulex*, and fish-hypolimnetic *D.pulex*. Each treatment was run in five replicates each of which contained two test organisms. *S.obliquus* was provided as fresh food with a concentration of 1.0 mg C L^{-1}. This experiment was carried out from 11th to 14th of July 2001.

2.4.2. DVM of *D.pulex* along with morphological response at varying food levels

On 12th to 17th March 2002, an experiment was carried out to compare the effect of different food levels on DVM and some morphological features of *Daphnia* in the presence of fish cue. Three different food concentrations which were 0.1, 0.4, 1.0 mg C L^{-1} for control and fish cue treatments were used. The concentration of 0.1 mg C L^{-1} was considered to be the starvation limit (Lampert, 1987b); therefore, it was considered as low food condition. The concentration of 0.4 mg C

L^{-1} was the concentration recorded in Lake Eymir from which the animals were brought. The concentration of 1.0 mg C L^{-1} was considered well above the limiting level; therefore, it was referred as high food condition. Each treatment was run in triplicates and each of which contained five individuals. As indicator of morphological adaptations, which included the distance from eye to head –named as head length- (x), eye to base –core body length- (y) and tail spine lenght (t) were measured before and after the experiment (Fig. 3). Impact of food levels and fish cue on morphological features was compared using total body length and the sum of head length and core body length (x+y+t and x+y, respectively). Additionally, x/y and t/y ratios were calculated to test the proportional changes in body length, to find out whether head length and tail length were linearly related to body length or not.

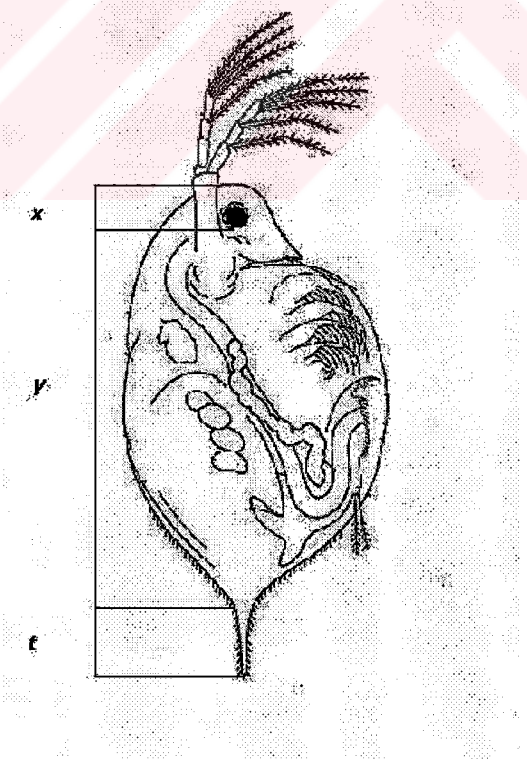

Figure 3. Schematic view of *Daphnia* (x: the distance from eye to head, named as head length; y: the distance from eye to base, named as core body length; t: tail spine length).

2.4.3. DVM experiments with varying bacterial density

The first experiment on 04th to 07th March 2001 was carried out to determine the effect of the fish cue on DVM of *D.pulex*. The general procedure was followed in the preparation of the treatments. Each treatment was represented with five replicates each of which included five *D.pulex* test animals. *S.obliquus* was provided as fresh food with a concentration of 2.0 mg C L^{-1}.

To test the impact of bacterial degradation on fish cue, four experiments with varying bacteria densities were carried out. These experiments consisted of five treatments, which included control (C), fish cue (F), filtered fish cue (FF), incubated fish cue (IF), and incubated-filtered fish cue (IFF). Each treatment was run with four replicates each of which included five *D.pulex* individuals. The general procedure was followed for the preparation of the treatments. The fish water was incubated for 24 h in the shaker. *S.obliquus* was provided as fresh food with a concentration of 1.0 mg C L^{-1}. The same experiment with five treatments was carried out on the 01st-06th November 2001, 23rd-28th January and 16th-18th May 2002. However, the experiment carried out on 25th to 28th July 2001 did not include filtered fish cue (FF) treatment. Hence each treatment included five replicates. Furthermore, fish cue was incubated for 8 h in the shaker to prepare IF and IFF treatments.

2.5. Preparation of the concentrated fresh food for the all DVM experiments

In order to prepare fresh food in desired concentration for *daphnids*, first carbon concentration of the *Scenedesmus obliquus* culture was estimated using the concentration of chlorophyll-a with to the conversion factor of 30: 1 C (μg) / chl-a

(µg) suggested by Reynolds (1984). The algal culture chlorophyll-a concentration was determined according to Jespersen & Christoffersen (1987). From the algae culture 10 ml was taken, and added to 90 ml of distilled water. Then the sample was filtered through a 47 mm Whatman GF/C glass-fiber filter paper. The filter paper was then placed in a plastic centrifuge tube with volume of 10 ml and 10 ml of 96% ethanol was added. The sample was incubated overnight at complete darkness. Following the incubation, the samples were centrifuged at 4000 rpm for 15 minutes. The absorbance of the supernatant was measured at 750 nm and 663 nm against an ethanol blank. The value at 750 nm corrects for any colloidal matter (Moss, 1967). Therefore, it was subtracted from the absorbance read at 663 nm. The chlorophyll-a concentration was calculated according to the equation below:

$$C_{\text{chlorophyll a}} = (11.0 \times (A_{663}-A_{750}) \times V_{eth}) / V_{\text{filt wat}}$$

Where: A_{663} is the absorbance at 663 nm

A_{750} is the absorbance at 750 nm

V_{eth} is the volume of ethanol blank in milliliters

$V_{\text{filt wat}}$ is the volume of the filtered water in liter

To obtain a more concentrated algal food, a method which was similar to the one used by Loose et al. (1993) (pers. comm. with Eric von Elert) was applied as described below. An appropriate amount of algae sample from the fresh culture was taken into the tube with a volume of 10 ml to centrifugation. The samples were centrifuged at the lowest speed of 2000 rpm to prevent the algae cells to burst. Eight milliliters of the centrifuged supernatant was carefully removed out using a micropipette. Then, the pellet was carefully and slowly re-suspended by adding 3 ml of fresh algae medium through suck & release using a micropipette.

Consequently, the concentration of the culture was doubled in half of the previous volume. As a last step, the concentrated algae culture was filtered through 35-μm of net to remove possible aggregations of algal cells that were inedible for *D. pulex* due to large size.

2.6. Statistical analyses

2.6.1. The method to determine outlier

Outlier is the value that does not fit to the general structure of the data. For determination of outlier, range that is not affected from the outlier values is determined and, the values out of this range are neglected. In our study, this range was determined as (F_L-1.5d_F, F_U +1.5d_F), the values out of this range were evaluated as outlier. F_L and F_U are the values that divide the data into quarter pieces, as 25% of the data below F_L and 25% of the data above F_U will be conserved. The d_f is the difference between F_L and F_U (F_U-F_L) and defines the data range. When assumed that the data is normally distributed, the possibility that having a value out of this data range is 0.00698 (0.698%) (Hoaglin, Mosteller & Tukey, 1982).

2.6.2. Analysis of variance

Repeated measures of ANOVA (SAS- Statistical Analysis System-SASOnlineDoc®, Version 8) was used to test the significance for all experiments except May 2002 experiment. Repeated measures of ANOVA analyses started from the second day of the observation for DVM experiments with varying bacteria density as IF and IFF treatments were introduced previous evening (for 1-

6 November, 23-28 January). One-way ANOVA (SPSS 10.0 for Windows) test was used to test the significance for the days that D.pulex individuals performed DVM. Two-way ANOVA and MANOVA were used for food experiment. Post hoc tests were performed using Tukey honestly significant difference-HSD.

2.7. Bacteria counts

Bacteria counts were performed simultaneously for the DVM experiments with fish cue vs. control, which was carried out from 4th to 7th of March 2001 and for DVM experiments with varying bacteria density, which consisted of three experiments carried out from 1st to 6th of November, 2001, 23rd to 28th of January, 2002 and 16th to 18th of May, 2002, except the experiment carried out between 25th to 28th of July, 2001. Enumeration of bacteria was carried out by Dr. Ayşegül Ozan by using standard culture techniques.

CHAPTER 3

RESULTS

3.1. Calibration of DVM experiments

3.1.1. Calibration of DVM experiments using different Daphnia species

Comparisons of the DVM performances of the two different *Daphnia* species revealed that both *D.pulex* and *D.magna* individuals responded significantly to the fish cue treatment (Repeated measures of ANOVA, P: 0.014, $F_{2, 69}$: 4.6) (Table 1). Additionally, the effects of the time and fish cue*time interaction on DVM of *D.pulex* were also highly significant (Repeated measures of ANOVA, P: < .0001, $F_{2, 138}$: 84; P: 0.0018, $F_{4, 138}$: 4.6) (Table 1). At the second day of the experiment, which was on the14th of July, *D.pulex* (F_{pulex}) and *D.magna* (F_{magna}) performed DVM in the fish cue treatments and, held the mean day-depths of 72 and 78 cm, respectively while the test animals in the control treatment stayed in the warm epilimnion (Fig.4). The DVM response remained the same during the following day with the mean day-depths of 70 and 93 cm, respectively. Difference between control and fish cue treatments was highly significant for both days (oneway-ANOVA, P: < .000, $F_{2, 102}$: 20; P: < .000, $F_{2, 73}$: 39, respectively) (Table 2). Post-hoc tests (Tukey-HSD) showed that the test animals in control treatment found in

the upper surface of the column, which were significantly different from the depth held by individuals of *D.pulex* and *D.magna*

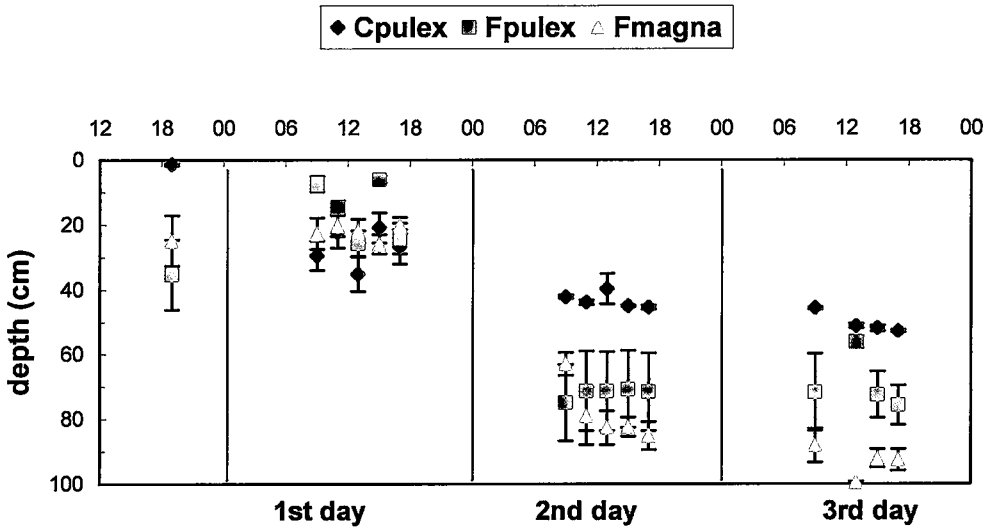

Figure 4. The mean day-depths (± C.I. 95%) of *D.pulex* vs *D.magna* individuals in response to the fish cue in an experiment carried out between 12th-15th June 2001 (Cpulex: *D.pulex* individuals in control treatment, Fpulex: *D.pulex* individuals in fish cue treatment, Fmagna: *D.magna* individuals in fish cue treatment).

in fish cue treatments both days (P: < .000) (Table 3). During the second day of the experiment, the individuals of *D.pulex* and *D.magna* in fish cue treatments resided in the hypolimnion that was not significantly different from each other (P: 0.613). However, on the third day, they moved significantly apart though the mean day-depths which were below 60 cm (P: < .000) (Table 3).

Table 1. Results of repeated measures of ANOVA on the effect of fish cue treatment on the DVM of *D.pulex* vs *D.magna* individuals collected from Lake Eymir and cultured in laboratory.

Treatment	df	F	P	Sig.
Fish cue	2	4.55	0.0140	*
Error	69			
Time	2	84.42	<.0001	***
Time*Fish cue	4	4.55	0.0018	**
Error (time)	168			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant

Table 2. Results of one-way ANOVA on the effect of fish cue treatment on the DVM of *D.pulex* vs *D.magna* individuals collected from Lake Eymir and cultured in laboratory.

Treatment		df	F	P	Sig.
Day2	**Fish cue**	2	19.836	.000	***
	Error	102			
Day3	**Fish cue**	2	39.301	.000	***
	Error	73			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant

Table 3. Results of post-hoc tests (Tukey-HSD) on the effect of fish cue treatment on the DVM of *D.pulex* vs *D.magna* individuals collected from Lake Eymir and cultured in laboratory.

Treatment		Control	Fpulex
Day 2	**F_{pulex}**	.000 ***	
	F_{magna}	.000 ***	.613 ns
Day3	**F_{pulex}**	.000 ***	
	F_{magna}	.000 ***	.000 ***

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant.

3.1.2. Calibration of DVM experiments using different *Daphnia* population

In the second experiment, which was undertaken on July 2001, the individuals of *D.pulex* collected from the hypolimnion and the epilimnion were used in the second calibration experiment to test upon clonal differences in *D.pulex*. Repeated measures of ANOVA revealed that they were significantly different in response to the fish cue effect (P: < .0001, $F_{3, 151}$: 95) (Table 4). Additionally, the effects of time and fish cue*time interaction on DVM of *D.pulex* were highly significant (Repeated measures of ANOVA, P: < .0001, $F_{2, 302}$: 90; P: < .0001, $F_{6, 302}$: 14) (Table 4).

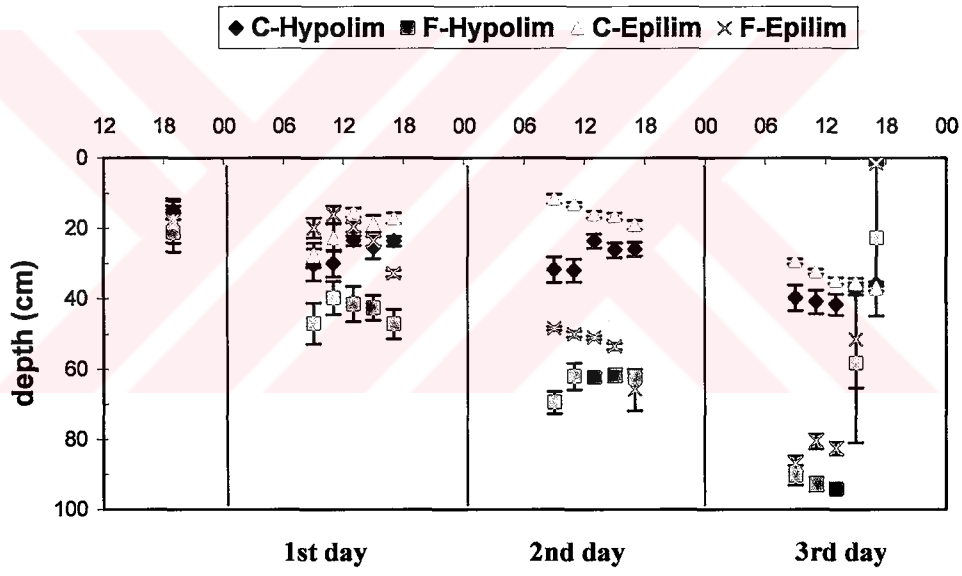

Figure 5. The mean day-depths (± C.I. 95%) of *D.pulex* individuals in response to the fish cue in an experiment carried out between 11-14 July 2001 (C-Hypolim: *D.pulex* individuals collected from hypolimnion in control treatment, F-Hypolim: *D.pulex* individuals collected from hypolimnion in fish cue treatment, C-Epilim: *D.pulex* individuals collected from epilimnion in control treatment, F-Epilim: *D.pulex* individuals collected from epilimnion in fish cue treatment).

Table 4. Results of repeated measures of ANOVA on the effect of fish cue treatment on the DVM of *D.pulex* individuals collected from the epilimnion vs the hypolimnion in Lake Eymir and cultured in the laboratory.

Treatment	df	F	P	Sig.
Fish cue	3	95.20	<.0001	***
Error	151			
Time	2	89.47	<.0001	***
Time*Fish cue	6	14.26	<.0001	***
Error (time)	302			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

At the second day of the experiment (13th of July), both hypolimnetic *D.pulex* and epilimnetic *D.pulex* individuals in the fish cue treatments had the mean day-depth of 64 and 54 cm, respectively. The individuals collected from hypolimnion in the fish cue treatment performed DVM at the second day whereas the individuals from epilimnion responded later. In control treatment, the both populations of *D.pulex* (epilimnetic and hypolimnetic) stayed in the epilimnion (Fig.5). Difference between treatments were highly significant for both days (One-way-ANOVA, P: < .000, $F_{3, 182}$: 349; P: < .000, $F_{3, 163}$: 20) (Table 5).

Table 5. Results of one-way ANOVA on the effect of fish cue treatment on the DVM of *D.pulex* individuals collected from epilimnion vs the hypolimnion in Lake Eymir and cultured in the laboratory.

Treatment		df	F	P	Sig.
Day2	**Fish cue**	3	348.507	.000	***
	Error	182			
Day3	**Fish cue**	3	19.935	.000	***
	Error	163			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Both the *D.pulex* individuals collected in the epilimnion and hypolimnion performed DVM regardless of their original habitat whereas the animals in control

treatments stayed in the upper layer of the columns (P: 0.003 & P: <. 000; P: <. 000, respectively) (Table 6). Although they both stayed below the thermocline, in the fish cue treatment the DVM response of the hypolimnetic *D.pulex* and epilimnetic *D.pulex* individuals differed from each other at a lower significance (P: 0.017) (Table 6) at the last day of the experiment.

Table 6. Results of post-hoc tests (Tukey-HSD) on the effect of fish cue treatment on the DVM of *D.pulex* individuals collected from epilimnion vs the hypolimnion in Lake Eymir and cultured in the laboratory.

	Control-Hypolimnetic	**Fish-Hypolimnetic**	**Control-Epilimnetic**
Day2			
Fish-Hypolimnetic	.000 ***		
Control-Epilimnetic	.000 ***	.000 ***	
Fish-Epilimnetic	.000 ***	.000 ***	.000 ***
Day3			
Fish-Hypolimnetic	.000 ***		
Control-Epilimnetic	.717 ns	.000 ***	
Fish-Epilimnetic	.003 **	.017 *	.000 ***

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant

3.1.3. DVM of *D.pulex* along with morphological response at varying food levels

The mean day-depths of the individuals in fish cue at the varying food levels differed significantly (Repeated measures of ANOVA, P: < .0001, $F_{1, 311}$:131; P:

0.008, $F_{2,\ 311}$: 5, respectively) (Table 7). Additionally, time, and interactions between time and the treatments on DVM of *D.pulex* were all highly significant (Table 7).

D.pulex individuals at 1 mg C l^{-1} (F1) food level performed a strong DVM in the presence of the fish cue onward the fourth day of the experiment, and held a mean day-depth of 76 cm whereas the individuals in the 0.4 and 0.1 mg C l^{-1} treatments stayed above the thermocline in the presence of fish cue (Fig. 6).

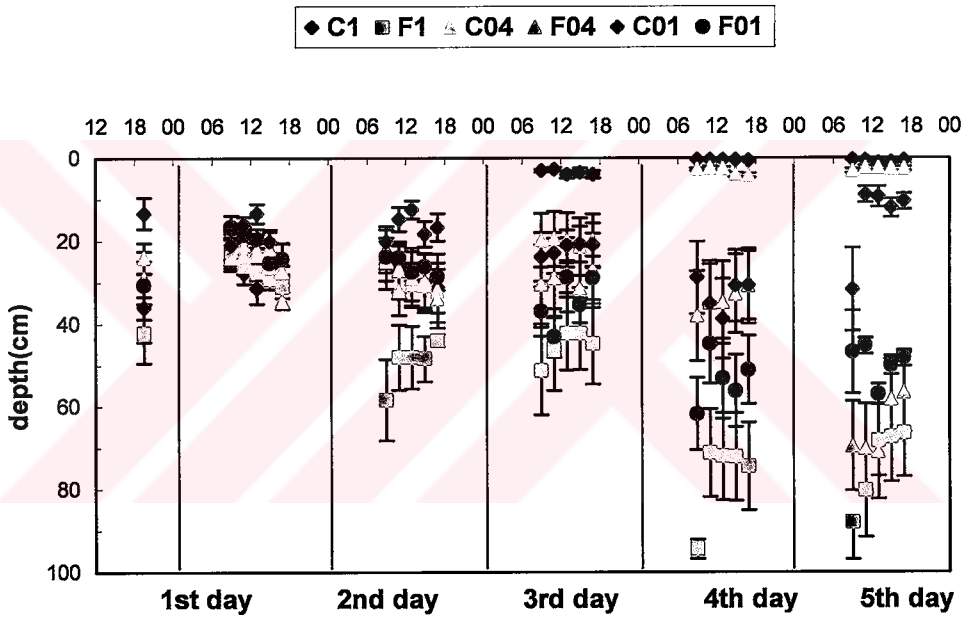

Figure 6. The mean day-depths (± C.I. 95%) of *D.pulex* individuals in response to the fish cue (F) and varying food concentration treatments in an experiment carried out between 12-17 March 2002 (C1: *D.pulex* individuals in control treatment with 1 mg Carbon L^{-1} food concentration, F1: *D.pulex* individuals in fish cue treatment with 1 mg Carbon L^{-1} food concentration , C04: *D.pulex* individuals in control treatment with 0.4 mg Carbon L^{-1} food concentration, F04: *D.pulex* individuals in fish cue treatment with 0.4 mg Carbon L^{-1} food concentration, C01: *D.pulex* individuals in control treatment with 0.1 mg Carbon L^{-1} food concentration and F01: *D.pulex* individuals in fish cue treatment with 0.1 mg Carbon L^{-1} food concentration).

Table 7. Results of repeated measures of ANOVA on the effect of treatment (absence or presence of fish cue) and food on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

Treatment	df	F	P	Sig.
Fish cue	1	130.67	<.0001	***
Food	2	4.90	0.0080	**
Fish cue*Food	2	2.33	0.0987	ns
Error	311			
Time	4	113.20	<.0001	***
Time*Fish cue	4	286.43	<.0001	***
Time*Food	8	55.94	<.0001	***
Time*Fish*Food	8	31.91	<.0001	***
Error (time)	1244			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant.

Table 8. Results of post-hoc tests (Tukey-HSD) on the effect of food on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory in fish cue treatments.

Treatment	F1	F04
Day4		
F04	.000 ***	
F01	.002 **	.003 **
Day5		
F04	.459 ns	
F01	.004 **	.021 *

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant.

Although the test animals in 0.1 mg C l^{-1} food concentration in the presence of fish cue (F0.1) stayed just above the thermocline with a mean-day-depth of 53 cm, they did not pass the thermocline the following day either. The individuals in 0.4 mg C l^{-1} food concentration in the presence of fish cue (F0.4) descended below the thermocline on the last day of the experiment and their mean day-depth was

65 cm. The mean day-depths recorded for F1 and F0.4 treatments did not reveal a significant difference at that day. (One-way ANOVA, P: 0.459) (Table 8). All the test animals in control treatments with 1, 0.4 and 0.1 mg C l^{-1} food levels (C1, C0.4 and C0.1) stayed in the epilimnion. Fish cue treatment and fish cue*food level interaction effects were highly significant at the fourth and fifth days (Two-way-ANOVA, P: < .000; $F_{1, 384}$: 205, $F_{2, 384}$: 29.7; $F_{1, 317}$: 371, $F_{2, 317}$: 17, respectively) (Table 9).

Table 9. Results of two-way ANOVA on the effect of fish cue and different food level treatments on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

Treatment	df	F	P	Sig.
Day4				
Fish cue	1	205.396	.000	***
Food	2	27.496	.000	***
Fish*Food	2	29.795	.000	***
Error	384			
Day5				
Fish cue	1	371.432	.000	***
Food	2	1.025	.360	ns
Fish*Food	2	17.268	.000	***
Error	317			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant.

The sum of head (x) and core body lenght (y) (x+y) (Fig. 7) and the total body length including the tail (t) (x+y+t) (Fig.8) significantly differed neither at the fish cue treatment (MANOVA, P: 0.094, $F_{1, 129}$: 2.847; P: 0.430, $F_{1, 129}$: 0.627, respectively) nor at the different food level (MANOVA, P: 0.393, $F_{2, 129}$: 0.939; P: 1.221, $F_{2, 129}$: 0.298, respectively) (Table 10a,b). However, the initial (x+y) and (x+y+t) lengths were significantly different from that of the final lengths

(MANOVA, P: .000, $F_{1, 129}$: 698; P: .000, $F_{1, 129}$: 479, respectively), whereas interaction effect between the treatments was not significant. On the other hand, the ratio of the head to core body length (x/y) significantly differed at the varying food level treatments in control treatment (MANOVA, P: 0.006, $F_{2, 129}$: 5.4) (Table 10c) (Fig.9). Additionally, time, time*food, food*fish cue and time*fish cue*food interactions were significant on the x/y ratio (MANOVA, P: .000, P: .006, P: .000, P: .000, respectively). The ratio of tail to core body length (t/y) did not significantly differ in response to fish cue and different food level treatments (MANOVA, P: 0.131, $F_{1, 128}$: 2.3; P: 0.6, $F_{2, 128}$: 0.52, respectively) (Table 10d) (Fig. 10). The t/y ratio significantly differed between the beginning and the end of the experiment whereas all the interaction effects were not significant (MANOVA, P: .000, $F_{1, 128}$: 155).

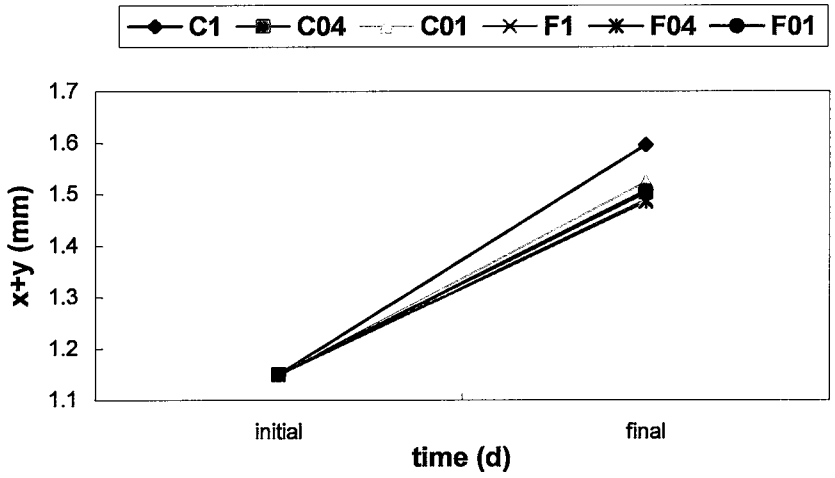

Figure 7. The changes in the sum of head (x) and core body length (y) of *D.pulex* individuals in response to fish cue and the varying food level treatments between the beginning and the end of the experiment carried out between 12-17 March 2002 (C1: *D.pulex* individuals in control treatment at 1 mg Carbon L^{-1} food concentration, F1: *D.pulex* individuals in fish cue treatment at 1 mg Carbon L^{-1} food concentration , C04: *D.pulex* individuals in control treatment at 0.4 mg Carbon L^{-1} food concentration, F04: *D.pulex* individuals in fish cue treatment at 0.4 mg Carbon L^{-1} food concentration, C01: *D.pulex* individuals in control treatment at 0.1 mg Carbon L^{-1} food concentration and F01: *D.pulex* individuals in fish cue treatment at 0.1 mg Carbon L^{-1} food concentration).

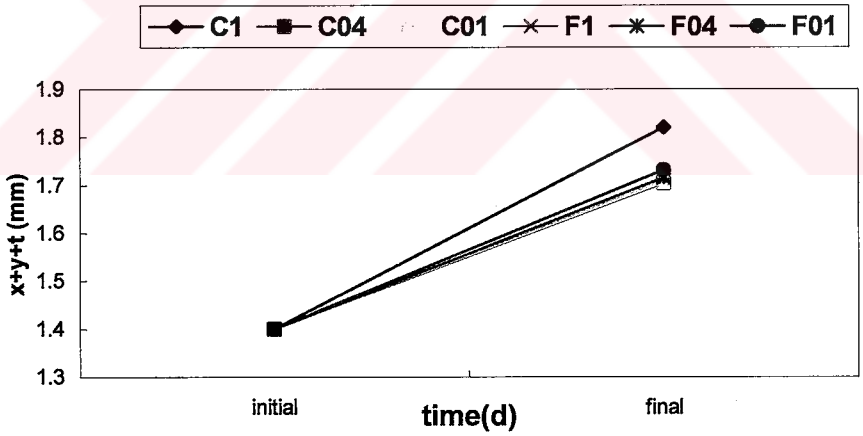

Figure 8. The change in total body length of *D.pulex* individuals in response to fish cue and the varying food level treatments between the beginning and the end of the experiment carried out between 12-17 March 2002 (C1: *D.pulex* individuals in control treatment at 1 mg Carbon L^{-1} food concentration, F1: *D.pulex* individuals in fish cue treatment at 1 mg Carbon L^{-1} food concentration , C04: *D.pulex* individuals in control treatment at 0.4 mg Carbon L^{-1} food concentration, F04: *D.pulex* individuals in fish cue treatment at 0.4 mg Carbon L^{-1} food concentration, C01: *D.pulex* individuals in control treatment at 0.1 mg Carbon L^{-1} food concentration and F01: *D.pulex* individuals in fish cue treatment at 0.1 mg Carbon L^{-1} food concentration).

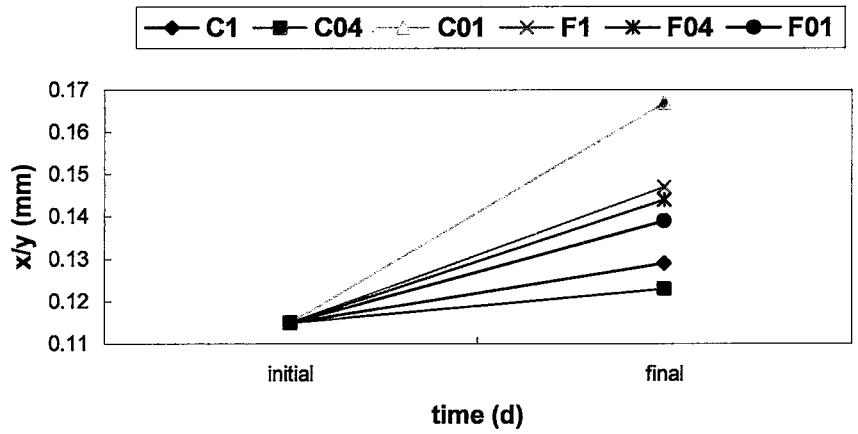

Figure 9. The change in the ratio of head length (x) to core body length (y) of *D.pulex* individuals in response to fish cue and the varying food level treatments between the beginning and the end of the experiment carried out between 12-17 March 2002 (C1: *D.pulex* individuals in control treatment at 1 mg Carbon L^{-1} food concentration, F1: *D.pulex* individuals in fish cue treatment at 1 mg Carbon L^{-1} food concentration , C04: *D.pulex* individuals in control treatment at 0.4 mg Carbon L^{-1} food concentration, F04: *D.pulex* individuals in fish cue treatment at 0.4 mg Carbon L^{-1} food concentration, C01: *D.pulex* individuals in control treatment at 0.1 mg Carbon L^{-1} food concentration and F01: *D.pulex* individuals in fish cue treatment at 0.1 mg Carbon L^{-1} food concentration).

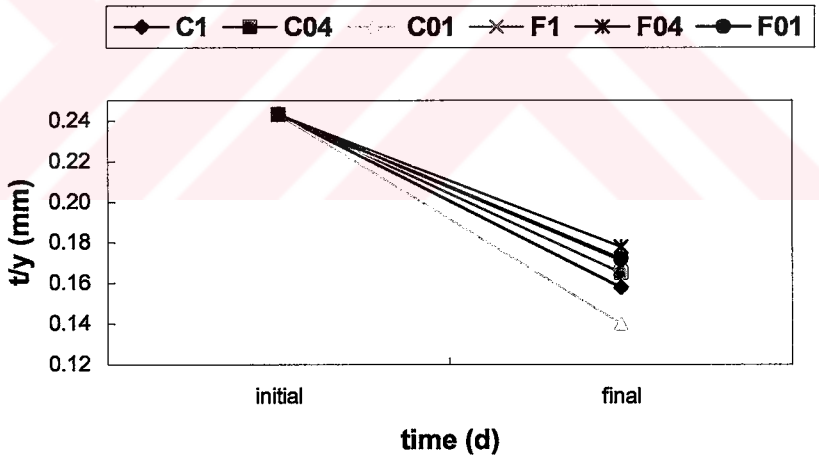

Figure 10. The change in the ratio of tail spine length (t) to core body length (y) of *D.pulex* individuals in response to fish cue and the varying food level treatments between the beginning and the end of the experiment carried out between 12-17 March 2002 (C1: *D.pulex* individuals in control treatment at 1 mg Carbon L^{-1} food concentration, F1: *D.pulex* individuals in fish cue treatment at 1 mg Carbon L^{-1} food concentration, C04: *D.pulex* individuals in control treatment at 0.4 mg Carbon L^{-1} food concentration, F04: *D.pulex* individuals in fish cue treatment at 0.4 mg Carbon L^{-1} food concentration, C01: *D.pulex* individuals in control treatment at 0.1 mg Carbon L^{-1} food concentration and F01: *D.pulex* individuals in fish cue treatment at 0.1 mg Carbon L^{-1} food concentration).

Table 10a. Results of MANOVA on the sum of head (x) and core body size (y) of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory at the fish cue and different food level treatments.

x+y	df	F	P	Sig.
Time (initial vs final)	1	697.8	.000	***
Fish cue	1	2.847	.094	ns
Food	2	0.939	.393	ns
Time*Fish cue	1	2.847	.094	ns
Time*Food	2	0.939	.393	ns
Fish cue*Food	2	1.057	.350	ns
Time*Fish cue*Food	2	1.057	.350	ns
Error	129			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant.

Table 10b. Results of MANOVA on the total body length of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory at the fish cue and food level treatments.

x+y+t	df	F	P	Sig.
Time (initial vs final)	1	479.4	.000	***
Fish cue	1	0.627	.430	ns
Food	2	1.221	.298	ns
Time*Fish cue	1	0.627	.430	ns
Time*Food	2	1.221	.298	ns
Fish cue*Food	2	1.887	.156	ns
Time*Fish cue*Food	2	1.887	.156	ns
Error	129			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant.

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DOKÜMANTASYON MERKEZİ

Table 10c. Results of MANOVA on the ratio of head (x) to core body size (y) of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory at the fish cue and food level treatments.

x/y	df	F	P	Sig.
Time (initial vs final)	1	102.4	.000	***
Fish cue	1	0.415	.520	ns
Food	2	5.36	.006	**
Time*Fish cue	1	0.415	.520	ns
Time*Food	2	5.36	.006	**
Fish cue*Food	2	9.631	.000	***
Time*Fish cue*Food	2	9.631	.000	***
Error	129			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant.

Table 10d. Results of MANOVA on the ratio of tail spine length (t) to core body size (y) of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory at the fish cue and food level treatments.

t/y	df	F	P	Sig.
Time (initial vs final)	1	154.8	.000	***
Fish cue	1	2.312	.131	ns
Food	2	0.516	.598	ns
Time*Fish cue	1	2.312	.131	ns
Time*Food	2	0.516	.598	ns
Fish cue*Food	2	0.256	.775	ns
Time*Fish cue*Food	2	0.256	.775	ns
Error	128			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant.

3.2. The DVM experiments with varying bacterial density

3.2.1. The DVM experiment with fish cue vs. control

The test animals in control and the fish cue treatment differed significantly according to their mean day-depths (Repeated measures of ANOVA, P: < .0001, $F_{1, 231}$: 81) (Table 11). Time and time*fish cue interactions on DVM of *D.pulex* were highly significant

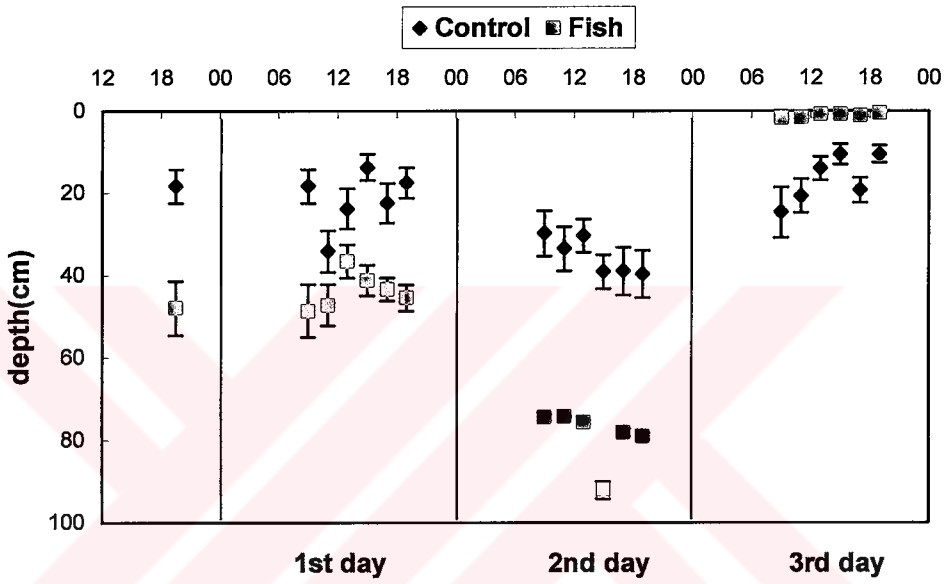

Figure 11. The mean day-depths (± C.I. 95%) of *D.pulex* individuals in response to the fish cue treatment in an experiment carried out between 04-07 March 2001 (Control: *D.pulex* individuals in control treatment, Fish: *D.pulex* individuals in fish cue treatment).

(Repeated measures of ANOVA, P: < .0001, $F_{2, 462}$: 529; P: < .0001, $F_{2, 462}$: 235, respectively) (Table 11). *D.pulex* individuals responded to the fish cue treatment at the second day of the experiment and performed a significant DVM while individuals in control treatment stayed in the epilimnion holding a mean day-depth of 35 cm (Fig.11).

The mean day-depth of the individuals in fish cue treatment was 80 cm. Comparison between the control and fish cue treatments revealed high

significance (One-way ANOVA, P: < .000, $F_{1, 242}$: 272) (Table 12). The strong DVM response in the fish cue treatment ended on the third day (Fig. 11).

Table 11. Results of repeated measures of ANOVA on the effect of fish cue treatment on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

Treatment	df	F	P	Sig.
Fish cue	1	80.61	<.0001	***
Error	231			
Time	2	528.82	<.0001	***
Time*Fish cue	2	234.82	<.0001	***
Error (time)	462			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Table 12. Results of one-way ANOVA on the effect of fish cue treatment on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

Treatment		df	F	P	Sig.
Day1	**Fish cue**	1	73.322	.000	***
	Error	293			
Day2	**Fish cue**	1	271.616	.000	***
	Error	242			
Day3	**Fish cue**	1	67.511	.000	***
	Error	231			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

3.2.2. The experiment carried out between 25th to 28th July, 2001

Repeated measures of ANOVA revealed high significance of the varying bacteria density in the presence of fish cue (P: < .0001, $F_{3, 371}$: 147) (Table 13). Furthermore, time and time*bacteria density interactions on DVM of *D.pulex* were

highly significant through the experiment (Repeated measures of ANOVA, P: < .0001, $F_{3, 1113}$: 183; P: < .0001, $F_{9, 1113}$: 244, respectively) (Table 13).

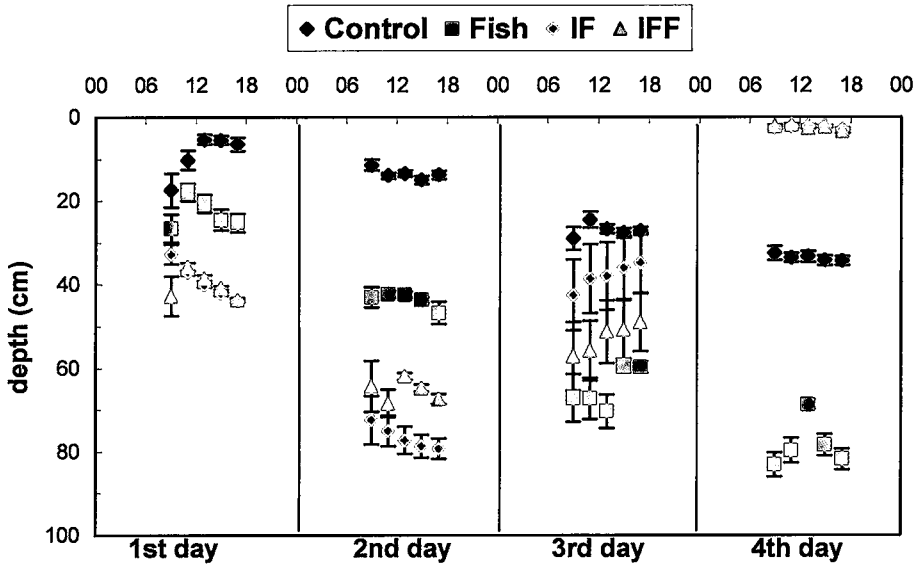

Figure 12. The mean day-depths (± C.I. 95%) of *D.pulex* individuals in response to the fish cue (F), incubated fish cue (IF) for 8 h. and incubated filtered fish cue (IFF) treatments along with control (C) in an experiment carried out between 25-28 July 2001.

Table 13. Results of repeated measures of ANOVA on the effect of fish cue treatment with varying bacteria densities on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

Treatment	df	F	P	Sig.
Bacteria density	3	146.77	<.0001	***
Error	371			
Time	3	182.74	<.0001	***
Time*Bacteria density	9	243.45	<.0001	***
Error (time)	1113			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Although the test animals in control treatment showed a tendency to move to the lower layer of the column towards the end of the experiment, they did not pass the thermocline and held a mean day-depth between 9.1 and 33.5 cm during the experiment (Fig. 12). On the second day of the experiment (26th July), *D.pulex*

individuals in incubated fish cue (IF) and incubated filtered fish cue (IFF) treatments performed DVM with a mean day-depth of 76 cm and 65 cm, respectively (Fig. 12). On the same day, the test animals in the fish cue (F), though they showed a tendency of moving towards the bottom, did not pass the thermocline and held a mean day-depth of 43 cm (Fig. 12). Post-Hoc tests

Table 14. Results of post-hoc tests (Tukey-HSD) on the effect of fish cue treatment with varying bacteria densities on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

	Control	F	IF
Day2			
F	.000 ***		
IF	.000 ***	.000 ***	
IFF	.000 ***	.000 ***	.000 ***
Day3			
F	.000 ***		
IF	.025 *	.000 ***	
IFF	.000 ***	.007 **	.000 ***
Day4			
F	.000 ***		
IF	.000 ***	.000 ***	
IFF	.000 ***	.000 ***	1.000 ns

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant.

(Tukey-HSD) revealed that at the second day, all the treatments were significantly different from each other (Table 14) (One-way ANOVA, P: < .000, $F_{3, 434}$: 534) (Table 15). Following day (27th July) animals in the fish cue treatment (F) performed DVM with a mean day-depth of 65 cm, while the animals in incubated fish cue (IF) and incubated filtered fish cue (IFF) treatments ascended and held a mean day-depth above the thermocline. The DVM response of the individuals in the fish cue treatment (F) continued the next day, whereas the animals in IF and IFF treatments moved to the upper epilimnion. Differences between the treatments continued to be significant the following days as well (P: < .000) (Table 14), except the lack of significant difference between IF and IFF on the last day (P: 1.000) (Table 14). The test animals in the control treatment remained above the thermocline that was significantly different from the other treatments during the experiment (Table 14) (Fig.12).

Table 15. Results of one-way ANOVA on the effect of fish cue treatment with varying bacteria densities on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

Treatment		df	F	P	Sig.
Day2	**Bacteria density**	3	534.169	.000	***
	Error	434			
Day3	**Bacteria density**	3	35.361	.000	***
	Error	455			
Day4	**Bacteria density**	3	2386.60	.000	***
	Error	381			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

3.2.3. The experiment carried out between 1st to 6th November, 2001

Comparisons of the treatments including control (C), fish cue (F), filtered fish cue (FF), incubated fish cue (IF) and incubated filtered fish cue (IFF) revealed that they were significantly different (Repeated measures of ANOVA, P: < .0001, $F_{4, 358}$: 37) (Table 16).

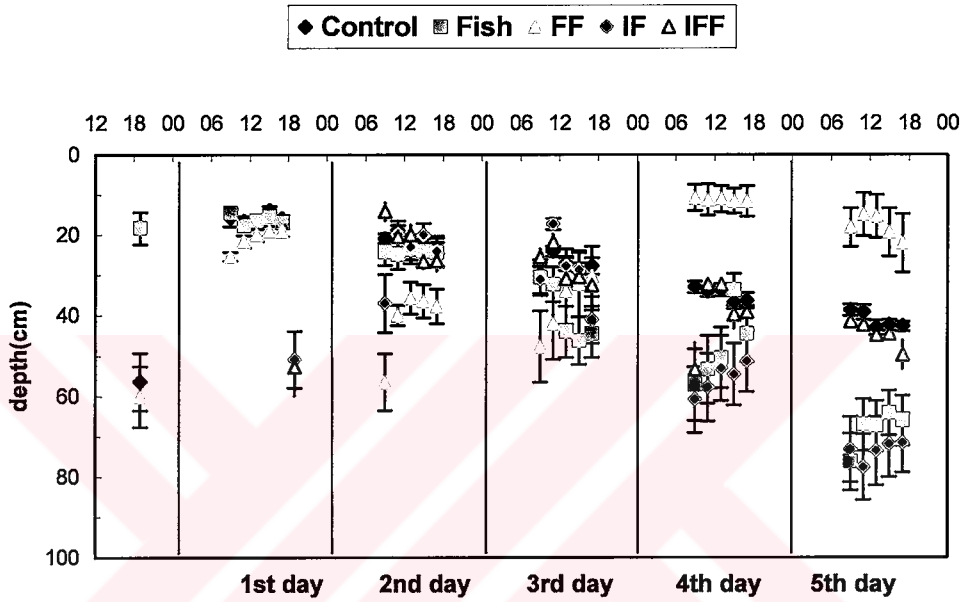

Figure 13. The mean day-depths (± C.I. 95%) of *D.pulex* individuals in response to the fish cue (F), filtered fish cue (FF), incubated fish cue (IF) for 24 h. and incubated filtered fish cue (IFF) treatments along with control (C) in an experiment carried out between 1-6 November 2001.

Table 16. Results of repeated measures of ANOVA on the effect of fish cue treatment with varying bacteria densities on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

Treatment	df	F	P	Sig.
Bacteria density	4	37.39	<.0001	***
Error	358			
Time	3	510.74	<.0001	***
Time*Bacteria density	12	76.98	<.0001	***
Error (time)	1074			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Furthermore, time and time*bacteria density interactions on DVM of *D.pulex* were significant (Repeated measures of ANOVA, P: < .0001, $F_{3, 1074}$: 511; $F_{12, 1074}$: 79, respectively) (Table 16). The animals in control treatment though showed a tendency to move to the lower layer of the column, did not pass the thermocline and held a mean day-depth between 21.5 and 41 cm throughout the experiment (Fig. 13). The animals in FF treatment were in the upper epilimnion though they had a small descend, but remained above the thermocline with a mean day-depth of 17.7 cm (Fig. 13). The individuals in the fish cue and incubated fish cue treatments (F and IF, respectively) responded late compared to the previous experiment. However, the individuals in the F and IF treatments performed DVM together at the fifth day of the experiment with a mean day-depth of 68 and 73.5 cm, respectively. Whereas individuals in IFF treatment did not performed DVM (Fig. 13). All the treatments were significantly different from each other on the fourth and fifth days (One-way-ANOVA, P: < .000, $F_{4, 430}$: 46; $F_{4, 374}$: 75, respectively) (Table 17). Additionally, at the fifth day, Post-Hoc test revealed that individuals in C and IFF treatments reacted in a similar way that animals did not perform DVM (One-way-ANOVA, P: 0.827; P: 0.482, respectively) (Table 18).

Table 17. Results of one-way ANOVA on the effect of fish cue treatment with varying bacteria densities on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

Treatment		df	F	P	Sig.
Day4	**Bacteria density**	4	46.224	.000	***
	Error	430			
Day5	**Bacteria density**	4	74.496	.000	***
	Error	374			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Table 18. Results of post-hoc tests (Tukey-HSD) on the effect of fish cue treatment with varying bacteria densities on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

	Control	F	FF	IF
Day4				
F	.001 **			
FF	.000 ***	.000 ***		
IF	.000 ***	.290 ns	.000 ***	
IFF	.515 ns	.129 ns	.000 ***	.000 ***
Day5				
F	.000 ***			
FF	.000 ***	.000 ***		
IF	.000 ***	.482 ns	.000 ***	
IFF	.827 ns	.000 ***	.000 ***	.000 ***

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant.

3.2.4. The experiment carried out between 23rd and 28th January, 2002

Comparisons between treatments including control (C), fish cue (F), filtered fish cue (FF), incubated fish cue (IF) and incubated filtered fish cue (IFF) revealed that they were significantly different (Repeated measures of ANOVA, P: < .0001, $F_{4, 363}$: 47) (Table 19). Furthermore, time and time*bacteria density interactions on DVM of *D.pulex* were also significantly different (Repeated measures of ANOVA, P: < .0001, $F_{3, 1089}$: 1191; $F_{12, 1089}$: 76, respectively) (Table 19).

The animals in control treatment did not pass the thermocline, and stayed in the epilimnion with a mean day-depth between 23.5 and 43.2 cm throughout the experiment as recorded in the previous July 2001 and November 2001

experiments (Fig. 14). Oneway ANOVA showed significant differences between groups for the fourth and fifth days (P: < .000, $F_{4, 424}$: 24; $F_{4, 399}$: 188, respectively) (Table 20). As opposed to the previous DVM experiments with varying bacteria densities, the test animals in FF treatment performed DVM on the fourth day of

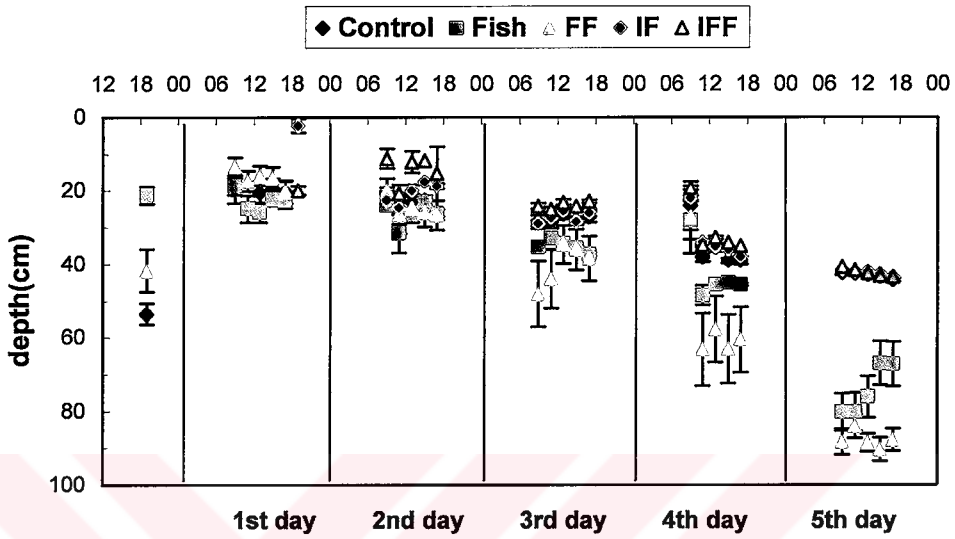

Figure 14. The mean day-depths (± C.I. 95%) of *D.pulex* individuals in response to the fish cue (F), filtered fish cue (FF), incubated fish cue (IF) for 24 h. and incubated filtered fish cue (IFF) treatments along with control (C) in an experiment carried out between 23-28 January 2002.

Table 19. Results of repeated measures of ANOVA on the effect of fish cue treatment with varying bacteria densities on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

Treatment	Df	F	P	Sig.
Bacteria density	4	46.65	<.0001	***
Error	363			
Time	3	1191.26	<.0001	***
Time*Bacteria density	12	76.20	<.0001	***
Error (time)	1089			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Table 20. Results of one-way ANOVA on the effect of fish cue treatment with varying bacteria densities on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

Treatment		Df	F	P	Sig.
Day4	**Bacteria density**	4	23.671	.000	***
	Error	424			
Day5	**Bacteria density**	4	187.610	.000	***
	Error	399			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Table 21. Results of post-hoc tests (Tukey-HSD) on the effect of fish cue treatment with varying bacteria densities on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

	Control	F	FF	IF
Day4				
F	.212 ns			
FF	.000 ***	.000 ***		
IF	.136 ns	.000 ***	.000 ***	
IFF	.405 ns	.001 **	.000 ***	.991 ns
Day5				
F	.000 ***			
FF	.000 ***	.000 ***		
IF	.993 ns	.000 ***	.000 ***	
IFF	.990 ns	.000 ***	.000 ***	1.000 ns

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant.

the experiment (27th January) with a mean day-depth of 62 cm, which was just below the thermocline. Its migration got deeper in the following day to 88 cm. The test animals in fish cue treatment (F) performed DVM a day later after FF on the fifth day of the experiment (28th of January) with a mean day-depth of 74 cm. Opposite to the previous varying bacteria densities experiments carried out on

July 2001 and November 2001, the test animals in the IF and IFF treatments did not performed DVM and stayed above the thermocline during the experiment. Post-Hoc test for the fifth day of the experiment revealed that the test animals in the IF, IFF treatments responded similarly with the control treatment (One-way-ANOVA, P: 0.993, P: 0.990, respectively) (Table 21). Additionally, the response of the animals in F and FF treatments were significantly different from the control, IF and IFF treatments (One-way-ANOVA, P: < .000) (Table 21).

3.2.5. The experiment carried out between 16th and 18th May, 2002

Comparisons between treatments including control (C), fish cue (F), filtered fish cue (FF), incubated fish cue (IF) and incubated filtered fish cue (IFF) revealed significant differences at the second day of the experiment (18th May) (One way-ANOVA, P: < .000, $F_{4, 430}$: 150) (Table 22). The test animals in the F and FF treatments performed DVM at the second day of the experiment, and held mean day-depths of 99.5 and 99 cm, respectively. Post-Hoc test did not reveal a significant difference between the F and FF treatments (One way-ANOVA, P: 0.999); however, they were significantly different from control, IF and IFF treatments (One way-ANOVA, P: < .000) as recorded in the previous January 2002 experiment (Table 23). The animals in control treatment did not descend below the thermocline and held a mean day-depth between 18 and 33 cm throughout the experiment similar to the previous varying bacteria density experiments (Fig. 15). Similar to the January 2002 experiment, the animals in IF and IFF treatments did

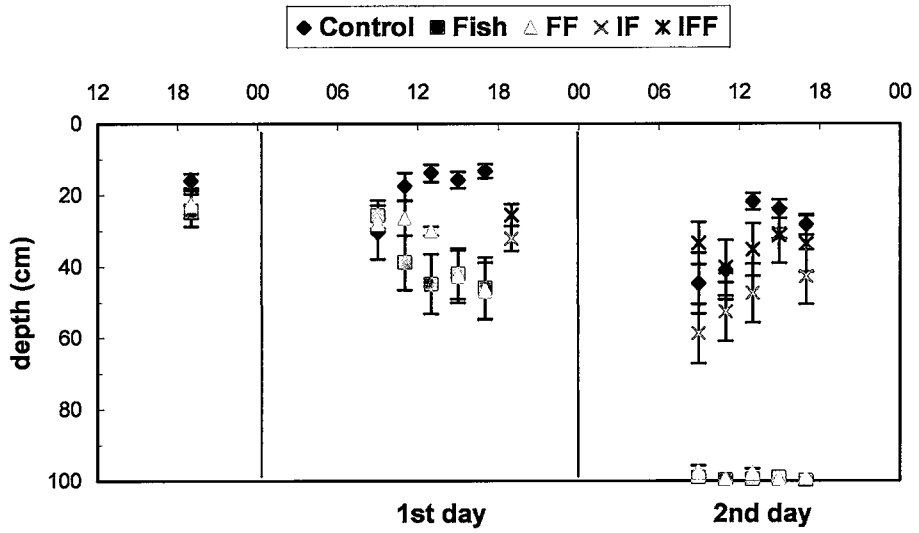

Figure 15. The mean day-depths (± C.I. 95%) of *D.pulex* individuals in response to the fish cue (F), filtered fish cue (FF), incubated fish cue (IF) for 24 h. and incubated filtered fish cue (IFF) treatments along with control (C) in an experiment carried out between 16-18 May 2002.

Table 22. Results of one-way ANOVA on the effect of fish cue treatment with varying bacteria densities) on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

Treatment		df	F	P	Sig.
Day2	**Bacteria density**	4	149.701	.000	***
	Error	430			

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Table 23. Results of post-hoc tests (Tukey-HSD) on the effect of fish cue treatment with varying bacteria densities on the DVM of *D.pulex* individuals collected from Lake Eymir and cultured in laboratory.

Treatment	**Control**	**F**	**FF**	**IF**
Day2				
F	.000 ***			
FF	.000 ***	1.000 ns		
IF	.001 **	.000 ***	.000 ***	
IFF	.988 ns	.000 ***	.000 ***	.004 **

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant.

not perform DVM, and held mean day-depths of 48 and 35 cm, respectively. Post-Hoc test revealed that the test animals in the IFF and control treatments responded similar that they were not significantly different from each other (One-way ANOVA, P: 0.998) (Table 23).

CHAPTER 4

DISCUSSION

In nature and as well as in controlled experiments, diel vertical migration of zooplankton as a predator-avoidance strategy has been recorded in a number of studies (Zaret & Suffern, 1976; Stich & Lampert, 1981; Gliwicz, 1986; Dodson, 1988a; Bollens & Frost, 1989; Dawidowicz et al., 1990; Neill, 1990; Ringelberg, 1991a; Dawidowicz, 1993; Loose, 1993a; De Meester et al., 1995; Ringelberg & Van Gool, 1998; De Meester & Weider, 1999; Von Elert & Pohnert, 2000; Lass et al., 2000; Pohnert & Von Elert, 2000; Spaak & Boersma, 2001).

DVM of the *Daphnia*, which is a key component of freshwater lake systems, is particularly well-studied (Dodson 1988a; Loose et al., 1993; De Meester et al., 1995; Spaak & Boersma, 2001). Predation risk by visual predators, mainly fish is today widely accepted as an ultimate cause of the DVM performed by zooplankton (Zaret & Suffern, 1976, Stich & Lampert, 1981, Gliwicz, 1986; Dodson, 1988a; Lampert 1989, 1993; Ringelberg et al., 1997; De Meester et al., 1999, Lass et al., 2000; Von Elert & Pohnert, 2000). Furthermore, many environmental factors such as temperature, food availability, transparency and oxygen concentration, which are considered as proxy factors influence the response of *Daphnia*; therefore, organisms determine strategies to cope with

unfavourable situations regarding the combination of these factors. (Johnsen & Jakobsen, 1987; Pijanowska & Dawidowicz, 1987; Ringelberg, 1991b; Haney, 1993; Loose & Dawidowicz, 1994; Gaso et al., 1995; Ringelberg et al., 1997; Ringelberg, 1999; Lass et al., 2000). As a pioneering study, Dodson (1988a) showed experimentally that a chemical substance exuded by fish, so called kairomone or cue, induced DVM in *Daphnia* within few hours. A number of following studies have revealed similar results on the stimulating effect of fish cue on the behavioural adaptation of *Daphnia* (Dodson, 1988b; Dawidowicz et al., 1990; Neill, 1990; Loose, 1993a; Pijanowska, 1993; Ringelberg at al., 1997; Matthew et al., 1999), as well as on the life-history traits (Slusarczyk, 1995; De Meester & Weider, 1999; Spaak et al., 2000; Hanazato et al., 2001; Slusarczyk, 2001; Weber, 2001) and on morphology (Dodson, 1988b; Dodson, 1989a; Spaak & Boersma, 1997; Boersma et al., 1998). However, only few recent studies revealed some physical and chemical features of the kairomone, which can be represented in general as water-soluble, released very quickly, non-volatile, low molecular weight of intermediate lipophilicity, stable under extreme conditions, non-protein, only vulnerable to bacterial degradation (Dodson 1988a; Larsson & Dodson 1993; Loose et al., 1993; Von Elert & Loose, 1996; Boriss et al., 1999; Forward & Rittschof, 1999, 2000; Von Elert & Pohnert 2000; Lass et al., 2001;). Several studies revealed that fish external mucus and its degradation products provoked DVM in some marine invertebrate larvae (Forward & Rittschof, 1999, 2000) whereas Loose et al. (1993) suggested that fish mucus did not induce DVM in a freshwater *Daphnia* species (see also Von Elert & Pohnert, 2000). Furthermore, it was suggested that bacteria associated with fish, probably

originating from fish mucus, produce kairomone responsible in the induction of DVM (Ringelberg & Van Gool, 1998). Whether the role of the bacteria associated with fish in the production of kairomones is not quite clear, it is widely accepted and shown that the kairomone released by predator is degraded by bacterial biological activity (Dodson 1988a; Loose et al., 1993; Ringelberg & Van Gool, 1995, 1998).

Studies on the characteristics of the kairomones becomes much more important as any result can help to analyze more precisely the community ecology of freshwater and marine organisms. Knowing the chemical nature of the kairomones would open the possibility for controlled experiments with many advantageous applications. For example, this may provide a new method for rapid assessment of fish population and/or activity in a lake (Larsson & Dodson 1993; Loose et al. 1993).

In this study, it was aimed to elaborate the effect of bacterial degradation on the activity of kairomone through varying bacterial density. All of the experiments statistically verified the significant effect of the presence of fish kairomone. The test individuals in the control and fish cue treatments were always recorded to differ behaviourally. While the individuals in the control treatment resided in the warm epilimnion, the individuals in the fish cue treatment performed strong DVM to cold hypolimnetic water. Furthermore, the DVM response was immediate in the fish cue treatment appearing within first two days that was in accordance with the previous experiments (Loose et al., 1993). On the other hand, the response of the individuals in the experiments carried out in winter & early spring (November 2001, January 2002 and March 2002) were slow that the DVM response took

place either on the fourth or fifth days. Such results indicated that there might have been a seasonality effect as indicated by Stibor & Lampert (2000) for the variance in the life-history traits of *Daphnia*. Furthermore, time and time-fish cue interaction effect were highly significant for all experiments.

Moreover, in this study, differences in DVM responses for the varying bacteria density, the varying food levels, and of among different *Daphnia* species and clones suggest that there is a decision-making mechanism in *Daphnia* as it was proposed previously by several researchers (Dini & Carpenter, 1988; Ringelberg, 1997; Sekino et al., 1999; Slusarczyk, 2001). There was a considerable variation in the amplitude of the DVM in fish cue treatments, while some individuals performed shorter migration, others moved down to the bottom of the water column as recorded in several studies (Dawidowicz & Loose, 1992; Loose & Dawidowicz, 1994). If DVM is a phenotypically induced plastic strategy, *Daphnia* seem to assess their chance for survival and successful reproduction. Such assessment is in accordance with the various conditions of danger of predation and food scarcity. This risk assessment strategy may lead individuals even within the same genotype to act differently in response to similar predation and environmental cues. In this study, as the *Daphnia pulex* cultures were not developed from the single mother, clonal differences were likely to have occured within the same cultures. However, the clonal selection was applied through culturing only the migrating animals that were used in all of the experiments.

During daytime, the small-sized zooplankters often remain higher in the water column compared to the large-sized which prefer to be in lower water column, have been recorded in different species comparisons (Hutchinson, 1967;

Pijanowska & Dawidowicz, 1987; Hays et al., 1994), as well as within species (Huntley & Brooks, 1982; Johnsen & Jakobsen, 1987). In this study, comparison of the DVM responses of the *Daphnia pulex* and *Daphnia magna* did not reveal any significant difference that both *D.magna* and *D.pulex* individuals performed DVM in response to the fish cue. However, *D.magna* resided slightly deeper than that of *D.pulex* (Fig.2). *D.magna* being the largest *Daphnia* species might have explained stronger response to fish cue, probably since *D.pulex* is the second largest *Daphnia* species it also performed a strong DVM. Differences between the species were not significant (Table 3). The two large species performing strong DVM are consistent with the previous observation by Pijanowska & Dawidowicz (1987) who showed that smaller *Daphnia* species occupied upper strata in the water column whereas the largest species occupied the deepest layer. Moreover, De Meester et al. (1995) suggested that clones differing in body size also differed in vertical distribution, with the largest clone residing at the greatest depth during the day since larger individuals are more vulnerable to visual predation than smaller ones.

De Meester & Weider (1999) showed that clones established from the epilimnion during the day were found to be smaller, both in the presence and absence of fish cue, than clones isolated from hypolimnion. The observation of small-bodied genotypes remained higher in the water column during the day than large-bodied ones is consistent with size-selective visual predation of fish in shaping *Daphnia* populations in lakes (Lampert, 1987c). The *D.pulex* collected from the hypolimnion (migrating clone) performed significantly more intense DVM in response to the fish cue than that of *D.pulex* clone collected from the epilimnion

(nonmigrating clone) (Table 5) (Fig. 3). The difference between the epilimnetic and hypolimnetic clones was rather related to the amplitude of migration. De Meester & Weider (1999) who utilized a similar methodology as in this study, showed that clones established from the epilimnion during the day were found to be smaller, both in the presence and absence of fish cue, than clones isolated from hypolimnion. Since the body size of the individuals used in the experiment was not measured, it was not obvious whether they also varied in size.

Several hypotheses have been proposed to determine the role of food availability in diel vertical migration. Dagg (1985) and Johnsen & Jakobsen (1987) suggested that migration occurs only when food is abundant; precisely only well-fed organisms can afford the cost of migration whereas it was also suggested that migration is more likely when food is limiting (Giguere and Dill, 1980; Hoenicke & Goldman, 1987; Dini & Carpenter, 1988,1992). Dini & Carpenter (1992) suggested that there was a hierarchy of factors in DVM, which identified fish predation as the primary forcing factor and food availability as the secondary factor. Therefore, if the predation by fish is strong, nonmigrating clones will be greatly reduced, or even eliminated. On the other hand, under less intense fish predation, this hierarchy invokes other factors such as food abundance and distribution to play important roles in determining daphnid behaviour. Furthermore, amplitude of vertical migration was affected by vertical heterogeneity in food availability (Pijanowska & Dawidowicz, 1987; Muluk & Beklioğlu, submitted). Van Gool & Ringelberg (1995) showed that *D.galeata x hyalina* exhibited a stronger phototactic behaviour to fish kairomones in the presence of food than in the absence of food (see also Muluk & Beklioğlu,

submitted). In this study, in the presence of fish kairomone, DVM was recorded at only 1 and 0.4 mg C l^{-1} food levels whereas at 0.1 mg C l^{-1} food level which is the starvation food limit (Lampert, 1987b), there was no DVM though the individuals showed strong tendency towards the hypolimnion. Furthermore, the individuals exposed to the highest food level resided at the deepest depth. They were aligned from the bottom of the tubes to the surface according to from the highest food availability to the lowest. The findings of this study are in accordance with the presence of stronger migrations when food is abundant in response to fish kairomone (Dagg, 1985; Johnsen & Jakobsen, 1987) and with Loose & Dawidowicz (1994) who showed the animals in the low food treatments staying higher in the columns.

Coupling of different anti-predator avoidance strategies has been argued in several studies. Some studies have suggested the coupling between predator-induced morphological and life-history changes (Parejko & Dodson, 1991) whereas several studies failed to show coupling of different strategies to predation pressure (Spitze, 1992; Lüning, 1994; Tollrian, 1995a; De Meester & Pijanowska, 1996; Boersma et al., 1998). In this study, during DVM response of *D.pulex* individuals to the food level and fish cue treatments, it was aimed to investigate whether there was a coupling between the behavioural response (DVM) and the morphological response. The sum of head and core body size (x+y), the total body length (x+y+t) and the ratio of tail to core body length (t/y) did not reveal any significant difference in response to fish kairomone at different food concentrations (Table 23). Moreover, the ratio of head to core body length (x/y) did not reveal any significance to the effect of fish kairomone whereas it revealed significant

difference to the different food treatment. In this study, there was no coupling between the morphological features and DVM of *Daphnia*. Moreover, the lack of coupling between the DVM and morphological features could be due to these two strategies being independent reactions. The lack of coupling between morphological and life-history changes in response to fish cue has been already recorded in *Daphnia* (De Meester, 1996; De Meester & Pijanowska, 1996). Furthermore, it was shown that predator induced different adaptation strategies could be uncoupled (Lüning, 1994; De Meester & Pijanowska, 1996; Boersma et al., 1998). On the other hand, the uncoupling between the morphometry and DVM recorded in this study might be due to the short period of time that the experiment took place. Five days may not have been enough to observe expected morphological adaptations compared to the previous studies, which mostly took about at least seven days. In addition, the test animals in control treatment at the lowest food level developed helmets in response to low food level. This unexpected response was previously shown to be induced by invertebrate predators (Dodson, 1988b; Dodson 1989b; Tollrian 1995a).

However, several studies recorded that fish predation induce changes in morphological structures (e.g. tail spine, neck teeth and helmet) (Zaret, 1980; Dodson, 1989b; Tollrian, 1995a). Some of the observations on morphological adaptations both in laboratory and field experiments revealed longer tail spines, smaller body length in response to fish cue (Dodson, 1988b; Dodson, 1989a; Barnhisel, 1991; Swaffar & O'Brien, 1996; Spaak & Boersma, 1997). Spaak & Boersma (1997) found that individuals in fish cue treatments induced longer tail spines at high food levels. Furthermore, Dodson (1988b) observed reduction in

morphological response of *D.retrocurva* under low food conditions. However, these studies explored the impact of fish predation on only a single predation-avoidance strategy which is morphological changes.

Few recent studies revealed some physical and chemical characteristics of fish kairomone (Dodson 1988a; Larsson & Dodson 1993; Loose et al., 1993; Von Elert & Loose, 1996; Ringelberg & Van Gool, 1998; Boriss et al., 1999; Forward & Rittschof, 1999, 2000; Von Elert & Pohnert 2000; Lass et al., 2001). Loose et al. (1993) experimentally showed that the activity of the fish cue disappeared within 24 h, in non-sterile conditions at 37 °C, whereas sterile treatment preserved its activity, indicating rapid bacterial biodegradation. Furthermore, several studies followed this finding attributed to the loss of kairomone activity to bacterial degradation. Hence, in this study, four DVM experiments with varying bacteria density were carried out to test the impact of bacterial degradation of fish cue and the response of *D.pulex* individuals to relative bacterial densities. In the March 2001 experiment, the highest bacterial growth was recorded in MS medium. Therefore, the counts of the following DVM experiments have been performed using MS media. The test animals performed in fish cue treatment (F) a strong DVM whereas the animals in control stayed in the epilimnion. However, the response of DVM in the fish cue treatment ceased at the third day of the experiment. The bacterial counts made at the beginning and end of the experiments provided a probable explanation (unpublished data on bacterial counts, A.Ozan). The bacterial count showed that the bacterial growth was extremely high in the fish cue treatment at the end of the experiment (Fig.16).

Hence, this might have led to the complete removal of the kairomone by bacterial degradation.

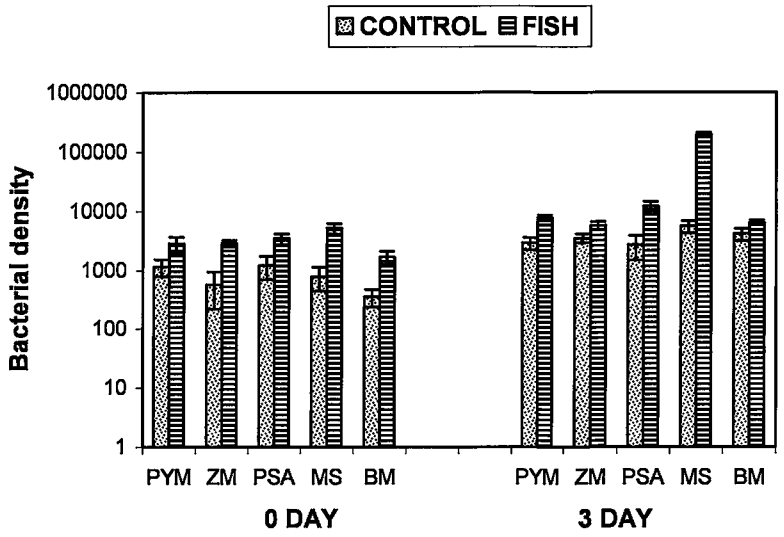

Figure 16. The mean (± SD) bacteria counts recorded in fish cue vs. control treatments during the beginning and the end of the DVM experiment with varying bacteria densities carried out in March 2001 using five different media.

Furthermore, the test animals in filtered fish cue treatments (FF) performed strong DVM, except in November 2001, and responded similar with the fish cue treatment. The bacterial counts for FF and IFF treatments showed that the bacteria removal was not completely efficient using the filtration method. Therefore, at the end of the November 2001 experiment, the bacterial growth in FF treatment was almost two-fold higher than in the fish cue treatment (Fig.17). Thus, the bacterial degradation might have cause to the lack of DVM in FF treatment in November 2001 experiment. Moreover, the test animals in the incubated-filtered fish cue treatments (IFF) did not perform DVM in November 2001, January 2002 and May 2002 experiments, and responded similar with control treatment that they were not significantly different from each other (Table 18,21,23). At the end of the November 2001 experiment, the bacterial growth in IFF treatment was almost

three-fold higher than in IF treatment. (Fig.17). This might have caused to bacterial biodegradation of the kairomone in IFF treatment. On the other hand, the responses to the varying bacteria density treatments (IF) can be grouped into two. Contrary to the expectation, the incubation of fish cue treatment to enhance bacterial density did not lead to bacterial degradation of kairomones and in turn, the DVM of *D.pulex* was strong in the July 2001 and November 2001 experiments. Whereas in January 2002 and May 2002 DVM experiments, *D.pulex* individuals did not perform DVM in the bacteria enrichment treatment probably due to the bacterial biodegradation. In January 2002, no DVM was observed in the bacteria enrichment treatment (IF) which had extremely high bacterial density (Fig. 18), in turn, this might have led to removal of the kairomone. On the contrary, the bacteria growth in the bacteria enrichment treatment (IF) was lowest in the November 2001 experiment where individuals performed a strong DVM. It was likely that the kairomone may not have been degraded due to low bacteria density. The bacterial growth in the bacteria enrichment treatment in May 2002 was moderate compared to the others; probably therefore, the response of *D.pulex* was intermediate; though individuals in the bacteria enrichment treatment showed tendency, they did not perform DVM. Therefore, these two different responses for the same bacteria enrichment treatment (IF) might have been due to inefficient biodegradation resulted from less bacterial growth to eliminate completely the effect of the fish cue resulted from the different bacterial growth. There might have an environment with lower kairomone which might have advertised predation pressure probably due to the

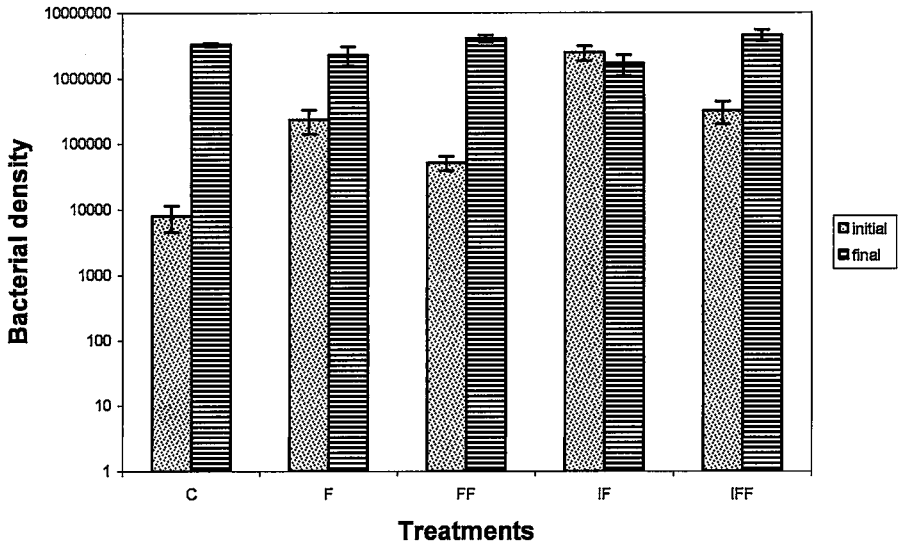

Figure 17. The mean (± SD) bacteria counts recorded in different treatments during the beginning and the end of the DVM experiment with varying bacteria densities carried out in November 2001,(C: control, F: fish cue, FF: filtered fish cue, IF: incubated filtered fish cue, IFF: incubated-filtered fish cue treatment).

partial degradation of the cue during incubation carried out on November and July 2001. However, bacteria enrichment treatment (IF) was prepared using the same method in all the experiments with incubation of the fish cue water in pre-sterilized volumetric flask for 24 h at 37 °C in the orbital shaker at 180 rpm. It is likely that the responsiveness of the *D.pulex* individuals might have weakened due to a lower kairomone activity due to partly biodegradation.

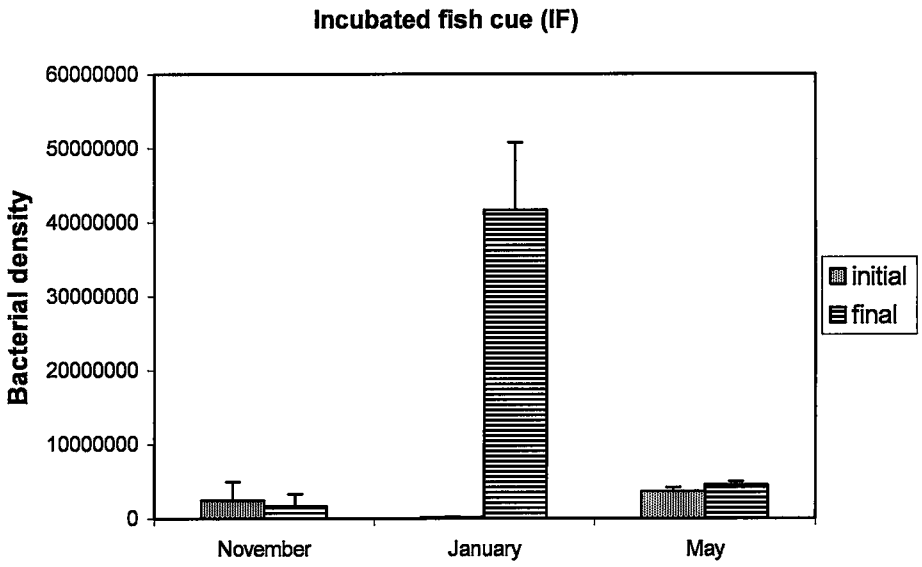

Figure 18. The mean (± SD) bacteria counts recorded in the bacteria enrichment treatment (IF) during the beginning and the end of the different DVM experiments with varying bacteria densities carried out in November 2001, January 2002 and May 2002.

Loose & Dawidowicz (1994) showed experimentally that above a threshold concentration of fish exudates, the strength of migration increased. In this study, the factors influencing the responsiveness of *D.pulex* to the same treatments may not have been attributed to the variations in food level, temperature or light intensity since all experiments were carried out under the same conditions in the controlled room. Furthermore, the fish cue treatment was prepared using the same density of fish (2 fish/10l) for all experiments, this may not have accounted for the differences. However, for the each experiment new fish brought from Lake Eymir were used. The kairomone concentration produced by fish might have changed seasonally and this might have caused different responses in the amplitude and strategies in the DVM. However, the results of this study to show the impact of varying bacterial density on the kairomone activity has not been conclusive since both the degradation and the lack of degradation responses were recorded in

response to the bacterial enrichment. Nevertheless, of all the experiment the test individuals in fish cue (F) treatment always performed DVM whereas the test individuals in control treatments stayed above the thermocline. These results show that the experiments carried out in this study achieved to show the impact of fish kairomones on DVM response of *D.pulex*. It can be concluded that this study was successful to simulate the effect of fish predation in the laboratory environment.

CHAPTER 5

CONCLUSION

In this study, it was aimed to investigate the effect of bacterial biodegradation of fish kairomone which induce DVM response in *Daphnia pulex*. To meet such goal, several DVM optimization experiments were carried out.

In the optimization experiments, comparison of the DVM responses of the *Daphnia pulex* and *Daphnia magna* revealed no significant difference. Both performed DVM in response to the fish cue. Moreover, the *D.pulex* collected from the hypolimnion (migrating clone) performed significantly more intense DVM in response to the fish cue than that of *D.pulex* clone collected from the epilimnion (nonmigrating clone) but the difference was rather related to the amplitude of migration. In the food optimization experiment, in the presence of fish kairomone, DVM of *D.pulex* was recorded at only 1 and 0.4 mg C l^{-1} food levels. They were aligned from the bottom of the tubes to the surface according to the highest food availability to the lowest. Furthermore, there was no coupling in responses to morphological features and DVM of *Daphnia* in the fish cue and varying food level treatments.

Four DVM experiments with varying bacteria density were carried out to test the impact of bacterial degradation on fish cue and the response of *D.pulex* individuals to the varying bacterial densities. The test individuals in fish cue (F)

treatment performed DVM whereas the test individuals in control treatments stayed above the thermocline in all experiments. The incubation of fish cue treatment (IF) to enhance bacterial density did not lead to bacterial degradation of kairomones in July 2001 and November 2001 experiments probably due to low bacterial growth. Whereas in January 2002 and May 2002 experiments, *D.pulex* individuals did not perform DVM in the bacteria enrichment treatment with higher bacterial growth, probably due to the bacterial biodegradation. These results may suggest a threshold bacterial growth for complete removal of fish kairomones might be needed. However, the experiments on varying bacterial density in removal of fish cue were not conclusive. Further experiments are suggested to be carried out that include complete removal of bacteria, maybe using antibiotics, would lead to conclusive results.

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APPENDIX A

PROTEOSE MEDIUM

General purpose freshwater medium suitable for axenic cultures. Modified Bristol's medium.

Preparation: to 940 ml of glass-distilled water, add 1.0 g proteose-peptone, 15.0 agar, and the following stock solutions:

ml	**stock solution**	**g/400 ml H_2O**
10	$NaNO_3$	10.0
10	$CaCl_2$ $2H_2O$	1.0
10	$MgSO_4$ $7H_2O$	3.0
10	K_2HPO_4	3.0
10	KH_2PO_4	7.0
10	NaCl	1.0