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Effects of probiotic as food additives on Nile catfish *Clarias lazera*.

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Abstract

Comparison of the concentrations of serum nitric oxide; malonaldhyde; total protein and its electrophoretical profile in Nile catfish *Clarias lazera* after supplementation with probiotic for 45 days either 1 time/week (W_1) all over the experiment or 3 times/week for the 1st two weeks then 1 time/week till the end of the experiment (W_3) were investigated. Probiotic supplementation affects on both serum nitric oxide and malonaldhyde. No effect of probiotic supplementation on serum total protein.

Twelve fractions were resolved of which 10 and 11 were the maximum numbers that appeared with consistency in females and males respectively. Polymorphism appeared in both males and females of the initial; control; W_1 and W_3 groups. Probiotic feeding altered the relative density of resolved serum protein fractions. In both W_1 and W_3 groups, the 9th fraction recorded higher values in both sexes, while fractions # 10 and 11 (in γ-globulin area) recorded lower values in both sexes. The relative densities of fractions # 5; 7 and 9-12 were significantly affected by probiotic supplementation.

Our study indicated that probiotic supplementation to fish diet is not a long-term effect and preferred to be added periodically according to farm management.

Key words: Probiotics, *Clarias lazera*, Serum total protein, Serum nitric oxide, Serum malonaldhyde.

Introduction

Today aquaculture is the fastest growing food-producing sector in the world with the greatest potential to meet the growing demand for aquatic food **(FAO, 2006)**. Globally, aquaculture is expanding into new directions, intensifying and diversifying **(Austin *et al*., 1995)**.

An excessive production of free radical causes an imbalance in the pro-oxidants/anti-oxidants system, leading to damage is termed oxidative stress **(Sies, 1991)**. Fish are exposed to natural or induced stressors, some of which induce reactive oxygen species (ROS) generation, which may promote morphological and/or physiological malfunctions.

All aerobic organisms possess antioxidant enzymes capable of preventing membrane cell damage, enzyme inactivation and nucleic acid alterations **(Aluwong *et al*., 2013)**. Presently, much attention has been focused on new approaches of anti-oxidant therapy by providing anti-oxidant enzymes.

Serum proteins are very complex and are in immense range of physiological importance. Knowledge of their roles in health and disease is of great importance to enzymologist and immunologist. Many reports are available on electrophoresis studies of serum fractions of healthy fish **(Rizkalla, 1988; Soliman *et al*., 1991; Rizkalla *et al*., 1999; Nabih *et al*., 2003; Akinwande *et al*., 2010 and Diyaware *et al*., 2012)**.

A new approach alternative method, that is gaining acceptance within the aquaculture industry, is by use of probiotic bacteria to control potential pathogens **(Gomez-Gil *et al*., 2000)**. Probiotics are microbial cell preparations or components of microbial cells of non-pathogenic bacteria or yeast species that help to equilibrate the intestinal microflora and are given to animals with the intention of improving health or production parameters **(Schrezenmeir and de Vrese, 2001)**. The exact mode of action of the probiotic bacteria has not been fully elucidated. Very little is known about the relative importance of these mechanisms **(Kesarcodi-Watson *et al*., 2008)**. Probiotic metabolic activities may have an antioxidant effect via the scavenging of oxidant compounds or the prevention of their generation in the intestine **(Azcárate-Peril *et al*. 2011)**. Probiotic exhibiting both antimicrobial and antioxidative benefits under different in vitro and in vivo conditions **(Truusalu *et al*., 2004; Songisepp *et al*., 2005; Järvenpää *et al*., 2007 and Rizkalla *et al*., 2013).**

The use of probiotics is an effort to contribute to the healthy development of aquacultured animals and to understand the role played in the immune and antioxidant systems. The probiotics are considered as a new strategy in feeding and health management in fish aquaculture practice **(Denev, 2008)**. Numerous studies have been focused on adult or juvenile fish antioxidant enzyme protection when ROS are generated by xenobiotics or fish exposure to pathogens **(Reyes-Becerril *et al*., 2008)**.

Despite its wider acceptance in aquaculture, many questions remain unanswered about its influence on fish physiology. The primary aim of the current study was to analyze and evaluate the effectiveness of probiotic mixture (consists of strains of lactic acid bacteria and yeast cells) on antioxidant enzymes and immuno response of Nile catfish *Clarias lazera*.

Material and Methods

Fish:

In May, healthy *Clarias lazera* (average length 40.3 ± 2.3 and 37.5 ± 3.5 cm for males and females respectively) were obtained from commercial fisheries in Giza governorate. The health status was examined immediately upon arrival according to the methods described by **Austin and Austin (1989)**. They were allowed to acclimatize the laboratory condition for 2 weeks. Experiment was carried out in glass aquaria with 150 L capacity filled with aerated de-chlorinated tap water and change of water was done once in 2 days intervals. Fishes were fed once daily with commercial diet (35 % protein, 3.08 % crude fat and 5.71 % crude fiber) at a fixed feeding rate 3 % of body

weight of fish. The feed given rate were adjusted at 7 days intervals. Glass aquaria were sterilized by potassium permanganate (4 mg/l) according to **Post (1987)**.

Probiotic:

Avi-Lution® was developed by Agri-King, Inc., Fulton, Illinois, USA. This probiotic contains 21.6 % live yeast cells (*Saccharomyces cerevisiae*, $5X10^9$ CFU/g); 16.8 % *Enterococcus faecium* bacteria ($1X10^{10}$ CFU/g); 2.5 % rapidly proliferating strain of lactic-acid producing bacteria (*Bacillus subtilis*, $5X10^9$ CFU/g); 8 % non-digestible oligosaccharides [inulin (92 %), glucose + fructose + sucrose (8 %)]; 27.7 % yeast culture (corn germ meal, cane molasses and corn syrup); 22.1 % calcium carbonate as carrier and 1.3 % other ingredients [paraffin oil (1.1 %), sodium silico aluminate (0.2 %)] . The recommended Avi-Lution® supplementation rate of 0.05 % contributes about 275 X 10^6 lactic acid bacteria/Kg of feed **(Hooge, 2000)**. The mixture is available as a granular premix.

Experimental design:

After the acclimatization period, blood was collected from ten fish (initial group). The experimental set-up was based on fed fish the commercial diet with probiotic "1 kg/ton ration" **(Agri-King, 2000)** for 45 days either one time/week all over the experiment (W_1 group) or three times/week for the 1^{st} two weeks then 1 time/week tell the end of experiment (W_3 group). Each group consists of 5 males and 5 females. Stocking density is 30 L/fish. The probiotic dose was dispersed into firm gelatinous capsules and pushed into the stomach of the fish. Fish of control group received the same gelatinous capsules free from probiotics.

Sampling:

Blood samples were drawn by cardiac puncture by using a sterile syringe. Blood allowed to clot at room temperature for 2 h. The tubes were kept slop at 4 °C for overnight, then centrifuged at 3000 ×g for 10 min and the supernatant serum was collected. The serum was stored immediately at -20 °C until analyses.

Biochemical serum parameters:

The initial and final biochemical analyses were estimated in fish sera. Total nitric oxide (NO) was measured colorimetrically at 540 nm as nitrite after reduction all nitrate to nitrite according to the method described by **Green *et al*. (1982)** and **Nims *et al*. (1995)**. Malonaldehyde (malondialdehyde, MDA) quantified colorimetrically at 532 nm according to the method described by **Okhawa *et al*. (1979)** and **Armstrong and Browne (1994)**. Total protein was carried out by Vitroscient diagnostic kits, Egypt, according to the method described by **Koller (1984)**.

Polyacrylamide gel electrophoretic analysis of serum proteins:

The freshly separated serum was fractionated using 7.5 % polyacrylamide gel electrophoresis **(Herzberg and Pasteur, 1975)**. Gels were stained by 1 % Amido black 10B and destained by 7 % acetic acid. The cleared gels were scanned and calculated according SynGene S. No. 17292*14518 sme*mpcs**.**

Statistical analysis:

The significant differences ($P < 0{\cdot}05$) between groups were determined by a one-way analysis of variance (ANOVA). Comparisons of sex were performed with *t*-tests. All statistics were performed using SPSS for Windows version 11.5 (SPSS Inc., Chicago, IL, USA).

Result and Discussion

Table (1) shows the values of serum biochemical parameters of *Clarias lazera* after 2 weeks acclimatization period. Significant sex differences were detected in which serum nitric oxide and total protein contents were lower in males than females. Table (2) demonstrated the significant changes in serum biochemical parameters after 45 days of probiotic feeding. Serum nitric oxide and malonaldhyde significantly ($P < 0.001$) increased and decreased respectively in both sexes of group W_1 compared to the control group. In W_3 group, serum malonaldhyde significantly decreased only in females. ANOVA test reported significant effect of probiotic supplementation on both serum nitric oxide and malonaldhyde. Sex difference disappeared in serum nitric oxide after probiotic feeding contrary to serum malonaldhyde in which significant ($P < 0.05$) sex difference was detected in W_3 group.

A net of pro-oxidants and the potency of an antioxidant defense system normally balanced in the body. Principal pro-oxidants are reactive species (including free radicals) divided into reactive oxygen species (ROS) and reactive nitrogen species (RNS) and they mediate the main effects of other pro-oxidative factors **(Sies, 1991; Halliwell and Gutteridge, 1999)**.The end products of lipid peroxidation are reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4- HNE), as natural bi-products. Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage **(Kalender *et al*., 2004)**.These explanation will appear in table (2).

A concentration of MDA in serum and tissues are generally used as a biomarker for radical-induced damage and endogenous lipid peroxidation **(Wang *et al*., 2008$_b$)**. The alteration appears in levels of the fish serum of MDA and nitric oxide are generally used as a biomarker for endogenous lipid peroxidation and radical-induced damage (table 2). These may caused by environmental burdens to which fish are exposed to during their growth. In addition, most importantly growth processes in early life is characterized with the generation of ROS through cellular division and apoptosis. This is because reactive oxygen are considered as the major mediators of oxygen cytotoxicity

and as important messengers stimulating cell division and manifesting cellular signaling effects **(Buetler *et al*., 2004)**.

Probiotics are effective against most various gram-positive pathogens and are known to be inhibitors of food spoilage microorganisms, including Listeria monocytogenes **(Pucci *et al*., 1988; Nielsen *et al*., 1990; Klaenhammer, 1993)**. It have the ability to enhancement of humoral and cellular arms of the immune system **(Takeshi and James, 2000)**. They enhancement of immune response and nutrition of host species through the production of supplemental digestive enzymes **(Abd El-Kader, 2009)**. Probiotic strains which are capable to limit excessive amounts of reactive radicals in vivo may contribute to prevent and control several diseases associated with oxidative stress **(Amaretti *et al*., 2013)**. probiotics may concretely enhance antioxidant defenses in the host, producing and releasing GSH and vitamins which are absorbed and distributed in the organism **(Spyropoulos *et al*., 2011)**.

The present work showed that serum total protein of *C. lazera* supplemented with probiotic (*Enterococcus faecium*; *Bacillus subtilis* bacteria and *Saccharomyces cerevisiae* yeast cells) significantly altered by sex (table 2). Serum total protein significantly ($P < 0.05$) decreased in males of both W_1 and W_3 groups compared to the control group, while increased ($P < 0.01$) in females of W_1 group only. No effect of probiotic supplementation on serum total protein was demonstrated with ANOVA test in both sexes. This observation was previously detected by **Rizkalla *et al*. (2013)** on the same fish species. Serum total protein level is measurable humoral component of the non-specific defense mechanism, and the elevated levels of the total serum protein and globulin indicates that fish are immunologically strong **(Nayak *et al*., 2004)**. **Wang *et al*. (2008_a)** indicated no remarkable difference in the total serum protein, albumin content and globulin concentration between the *E. faecium* fed tilapia (*Oreochromis niloticus*) and the control fish. **Kumar *et al*. (2006)** described an increase in the serum protein and globulin levels in the *B. subtilis* feeding major carp and thought to be associated with a stronger innate response in fish. **Mahmoud (2012)** mentioned that the highest value of plasma albumin was recorded in fish fed on a diet supplemented with *Lactobacillus acidophilus*, while the highest value of globulin was achieved with fish group fed on a diet supplemented with bacterial mixture (*Lactobacillus acidophilus*, *Streptococcus thermophilus* and *Bifidobacterium bifiduim*) and yeast (*S. cerevisiae*). On the other hand, the lowest values of total protein and albumin were recorded in fish group fed on a diet supplemented with *B. bifiduim*. **Khattab *et al*. (2007)** mentioned that the plasma total protein was significantly decreased in *O. niloticus* fed with diet containing mixture of *Micrococcus luteus* and *Pseudomonas* species as probiotic. Also, **Abu-Elala *et al*. (2013)** revealed significant decrease in serum total protein; albumin and α-globulin of *O. niloticus* treated with yeast (*S. cerevisiae*), while β-globulin significantly increased and γ-globulin value presented a non significant difference compared to the control group. Working on *Catla catla*, **Bandyopadhyay and Das**

Mohapatra (2009) supported the use of probiotic (*Bacillus circulans*) to be an important immunostimulant in *Catla catla* and concluded that the lowest significant A/G ratio observed in sera of fish fed *B. circulans* might be due to increased lymphocyte proliferation and subsequent immunoglobulin production. **Parthasarathy *et al*. (2012)** reported that plasma protein is directly correlated to that of the total crude protein of fish fed the probiotic bacteria (*Lactobacillus plantarum*; *Bacillus megaterium* and combined *L. plantarum* & *B. megaterium*) and the probiotic bacteria can eliminate the pathogenic bacteria which cause disease in fish.

The electrophoretic patterns of sera of these initial and three groups of fish revealed distinct bands. In the four groups as many as 12 fractions were resolved of which 10 and 11 were the maximum numbers that appeared with consistency in females and males respectively (tables 1 & 3). These fractions were numbered sequentially according to their relative mobility i.e. the most rapidly migrating being No. 1. The numbering was for the sake of convenience. The serum proteins were separated on the same electrophoretic run. By this it was possible to make direct comparison of the protein bands. The fractions were classified into three main groups (tables 1 & 3): more mobile fractions (# 3 - 5); mid mobile fractions (# 6 - 9); less mobile fractions (# 10 - 11) in addition to the fractions # 1 & 2 (fastest) and fraction # 12 which is the closest one to the point of application. Fractions # 1 & 2 in serum of *Clarias lazera* are the fast migrating like prealbumin and albumin. Three fractions (# 3 – 5) had occupied the position of α-globulins. At the β-globulin region, four large fractions (# 6 – 9) were observed. Above that are the slow migrating fractions (# 10 & 11) equal to γ-globulins. The last fraction (# 12) near the resolving gel is the highest molecular weight one and that of IgM.

The 3rd band was absent in males of the initial; W_1 and W_3 groups, while the 4th band was not observed in males of the control group. In females, proteinograms of initial and control groups did not show 1st and 4th bands. Only 2nd band was absent in W_1 and W_3 groups in addition to 6th band in W_3 group. All the groups had the 5th; 7th to 12th bands. The consistent appearance of these bands corresponding to the α-; β- and γ-globulin fractions of serum protein. Working on the same fish species, **Rizkalla (1988)** mentioned that as many as 13 bands were discernible in some gels although 10 was the maximum number that appeared with consistency. **Rizkalla *et al*. (1999); Soliman *et al*. (1991)** and **Nabih *et al*., 2003)** reported to 10; 11 and 12 fractions respectively. These variations of electrophoretic patterns might be a result of changes in the physiological and environmental conditions. 13 and 16 protein bands were resolved by **Diyaware *et al*. (2012)** from serum of *C. anguillaris* and **Akinwande *et al*. (2010)** from *C. gariepinus, C. anguillaris* respectively. This variation could be due to the differences in the species and the environment where the experimental fish species were collected.

In the present study, the 1st and 2nd fractions (highly migrated bands that represent prealbumin and albumin fractions) showed clear polymorphism (tables 1 & 3).

De Smet *et al*., (1998) reported that serum albumin in some fish species may exist in minute quantities, while sera of other fish species may entirely lack it. This observation was supported by **Shamsudddin *et al*. (2011)** that no albumin like large fraction in the sera of fishes and the largest fractions were seen at the level of β-globulins. Albumin has transport and carrier functions, participates in osmoregulation of blood and is a reserve protein of the organism and an intensive supply of food resulted in high levels of this fraction **(Vlasov, 1974)**. For that reason, fractions # 1 and 2 represent 9.9 % (control group) – 13.1 % (W_3 group) of the total fractions after probiotic supplementation (table 3). **Boon *et al*. (1987)** suggested that the plasma protein fraction # 1 is an indicator for the health status of young *C. gariepinus*. **Rizkalla *et al*. (1999)** found that the intensities of the more mobile fractions were significantly lower in the +ve non-toxogenic *Clostridium perfringens* infected *C. lazera* than in the –ve group.

The distal fractions (# 3 – 5) could be described as α-globulins. Fraction # 5 showed significant lower percentages in W_1 and W_3 (male only) groups compared to control (table 3). These two groups significantly affected by probiotic feeding ($P < 0.01$ and 0.05 for male and female respectively, ANOVA test). **Rónyai *et al*. (1982)** said that the increase in the α-globulin fraction was recorded in diseased individuals.

According to their mobility, the mid fractions (# 6 – 9) could be described as β-globulins (tables 1 & 3). The presence of moderately molecular weight proteins like of β-globulins, haptoglobins and transferrins in *Channa punctata* blood serum were reported by **Gaikwad *et al*., (2012)**. The sum of the relative intensities of fractions # 6 – 9 (table 3) revealed that probiotic supplementation raises this sum from 52.0 and 54.7 % for males and females respectively of control group to 61.7 and 59.9 % respectively of W_1 group and to 61.6 % for males and 55.7 % (without fraction # 6) for females of W_3 group. This indicates that probiotic affects on transferrins in the β-globulin protein which transports iron and has antioxidant properties **(Sheila and Brock, 1992)**.

The 10^{th}, 11^{th} and 12^{th} fractions which are the least mobile fractions could be assessed as the gamma globulins. *C. lazera* supplemented with probiotic had a significantly ($P < 0.001$) lower percentages of the three fractions in W_1 and W_3 groups (except females in W_3 group) than that of the control group (table 3). The fraction of the γ-globulin includes immunoglobulins which are essential for the formation of antibodies. A higher percentage of the γ-globulin fraction was found in diseased fish **(Rónyai *et al*., 1982)**. **Rizkalla *et al*. (1999)** recorded that the *C. lazera* that had +ve non-toxogenic *Clostridium perfringens* in its intestine showed significant increases in the serum protein γ-globulin. The last decade have been demonstrated that many probiotic agents stimulate certain cellular and humoral functions of the immune system and have a positive effect on fish health **(Panigrahi *et al*., 2004, 2005; Balcázar *et al*., 2007)**. Supplementation of Nile tilapia, *Oreochromis niloticus*, with dietary bacterial strain, *Enteroccus faecium* **(Wang *et al*., 2008_a)** and mixture of bacteria *Bacillus subtilis* & *Lactobucillus plantarum* and yeast *Sacchromyces cerevisiae* **(El-Ezabi *et al*., 2011)**

as probiotics succeeded in improving certain immunological parameters. Also, **Parthasarathy *et al*. (2012)** showed - by immuno electrophoresis - that the serum of the *Catla catla* which was under the treatment of probiotic bacteria (*L. plantarum* & *Bacillus megaterium*) developed antibodies against the pathogen *Aeromonas hydrophila*. Working on rainbow trout, *Oncorhynchus mykiss*, **Nikoskelainen *et al*. (2003); Panigrahi *et al*. (2004) and Tukmechi *et al*. (2007)** reported an increase in total serum immunoglobulin levels of fish fed diet containing *Lactobacillus rhamnosus*. Yeast cells have been found to be effective in increasing the serum immunoglobulin IgM level of seabream, *Sparus aurata*, by its supplementation to the diet as an immunostimulatant **(Cuesta *et al*., 2004)**.This conferms that the probiotic can stimulate the antibody production in fish **(Panigrahi *et al*., 2004)**. **Panigrahi *et al*. (2005)** reported that the plasma total Ig level of all the probiotic fed rainbow trout groups irrespective of the form of lactic acid bacteria (LAB) showed an increasing trend at 20 days. Thereafter, the level of total immunoglobulin decreased in all LAB fed groups reaching levels similar to that in control group of fish at 30 days suggesting that the stimulation of the immune system was a short-term phenomenon attributable to the probiotic. Upon withdrawal of the probiotic diets, the LAB disappeared from the intestine and the elevated immune parameters returned to the prefed level. Upon the observation of **Panigrahi *et al*. (2005)** we can concluded that the decreasing level of γ-globulin fractions in the present work may be attributed to the long experimental (45 days) period.

Conclusion:

This study showed that antioxidant enzymes and immuno response of Nile catfish *Clarias lazera* changes after probiotic supplementation. These changes were observed as differences in the nitric oxide; malonaldhyde and the electrophoretic profile of serum protein by means of preventing oxidative stress, and by maintaining lipid peroxidase and nitric acid. This study supports the hypothesis that selected probiotic strains can underlay the enhancement of cellular antioxidant defenses in the host. Our study indicated that probiotic supplementation to fish diet is not a long-term effect and preferred to be added periodically according to farm management.

Table (1): Serum parameters of *Clarias lazera* after 2 weeks acclimatization period (initial group).

Parameters			Male	Female
			Mean ± S.D.	Mean ± S.D.
Nitric Oxide (µmol/l)			0.104 0.002	0.165 0.055
			P < 0.05	
Malonaldhyde (µmol/l)			2.672 0.634	2.080 0.752
Total Protein (g/dl)			3.204 0.360	4.442 0.637
			P < 0.01	
Electrophoretic Protein Fractions (Relative Density)	More mobile	F_1	7.92 0.040	N.D.
		F_2	4.23 0.521	7.95 1.109
			P < 0.001	
		F_3	N.D.	11.06 0.744
		F_4	9.61 1.036	N.D.
		F_5	16.64 2.744	11.96 2.838
			P < 0.05	
	Mid mobile	F_6	9.20 0.249	13.32 4.516
		F_7	5.70 0.646	10.52 5.190
		F_8	10.23 2.873	12.17 1.719
		F_9	24.32 0.728	19.51 2.461
			P < 0.01	
	Less mobile	F_{10}	6.33 0.875	6.57 0.613
		F_{11}	3.53 0.305	5.04 1.033
			P < 0.05	
		F_{12}	2.28 0.123	1.89 0.401

Number of samples/group = 5 N.D.: Not detected

Table (2): Serum total protein, nitric oxide and malonaldhyde levels of *Clarias lazera* fed on probiotic either 1 time/week for 45 days (W_1) or 3 times/week for 14 days then 1 time/week for the rest of 45 days (W_3).

Parameters	Sex	Control		W_1		W_3		ANOVA
		Mean	± S.D.	Mean	± S.D.	Mean	± S.D.	(P <)
Nitric Oxide (µmol/l)	♂	0.210	0.010	0.318	0.008^{***}	0.189	0.036	0.01
	♀	0.144	0.028^{b}	0.320	0.071^{***}	0.218	0.076	0.05
Malonaldhyde (µmol/l)	♂	2.272	0.083	0.978	0.008^{***}	2.012	0.246	0.01
	♀	4.924	1.344^{b}	1.200	0.306^{***}	1.660	0.157^{***a}	0.01
Total Protein (g/dl)	♂	3.475	0.083	3.293	0.093^{*}	2.749	0.529^{*}	N.S.
	♀	3.589	0.349	4.252	0.115^{**c}	3.780	0.706^{a}	N.S.

Significant difference to respective control: *: P < 0.05; **; P < 0.01 and ***: P < 0.001

Significant values between the two sexes in the same group are represented by:

a: P < 0.05, b: P < 0.01 and c: P < 0.001.

Number of samples/group = 5 N.S.: Not significant

Table (3): Relative density of serum electrophoretic protein fractions of *Clarias lazera* fed on probiotic either 1 time/week for 45 days (W_1) or 3 times/week for 14 days then 1 time/week for the rest of 45 days (W_3).

Fraction		Sex	Control		W_1		W_3		ANOVA
			Mean	± S.D.	Mean	± S.D.	Mean	± S.D.	(P <)
More mobile	F_1	♂	6.67	0.529	7.43	0.492*	6.42	0.573	N.S.
		♀	N.D.		7.27	1.214	7.24	1.713	
	F_2	♂	3.18	0.201	4.54	0.297***	6.68	0.430***	0.01
		♀	7.94	0.930^{c}	N.D.		N.D.		
	F_3	♂	10.75	0.026	N.D.		N.D.		
		♀	10.28	1.973	12.29	0.655	9.12	1.968	N.S.
	F_4	♂	N.D.		11.11	0.665	10.89	3.912	
		♀	N.D.		5.32	0.859^{c}	5.07	0.940^{a}	
	F_5	♂	13.39	1.733	8.41	0.417***	7.69	0.468***	0.01
		♀	13.37	0.636	8.55	0.351***	13.44	2.205^{c}	0.05
Mid mobile	F_6	♂	9.40	0.587	8.78	0.347	10.55	0.203**	0.05
		♀	12.09	2.128^{a}	9.59	0.742*	N.D.		
	F_7	♂	11.76	0.888	6.58	0.272***	7.22	1.143***	0.01
		♀	10.67	3.209	6.75	0.509*	15.48	2.415^{*c}	0.05
	F_8	♂	9.95	0.439	19.17	0.473***	17.85	7.378*	N.S.
		♀	13.69	1.615^{b}	15.04	3.838^{a}	15.39	2.440	N.S.
	F_9	♂	20.89	0.458	27.21	0.172***	26.00	1.826***	0.01
		♀	18.20	0.610^{c}	28.51	2.126***	24.80	3.135**	0.01
Less mobile	F_{10}	♂	7.46	0.304	2.62	0.145***	2.29	0.110***	0.01
		♀	6.25	0.764^{a}	2.06	0.435^{***a}	2.78	0.823***	0.01
	F_{11}	♂	3.48	0.072	2.79	0.230***	3.10	0.104***	0.05
		♀	5.73	0.696^{c}	3.72	0.536^{***b}	2.29	0.479^{***b}	0.01
	F_{12}	♂	3.06	0.190	1.37	0.047***	1.31	0.246***	0.01
		♀	1.78	0.548^{b}	0.90	0.272^{*b}	4.39	0.359^{***c}	0.01

See footnote table 2 N.D.: Not detected

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