North-Western Journal of Zoology
P-ISSN: 1584-9074, E-ISSN: 1843-5629

Vol. 6, No. 2, 2010, pp.162-171
Article No.: 061115

Reproduction in the brine shrimp *Artemia* Leach, 1819 (Branchiopoda, Anostraca) from South India: Laboratory cross fertility tests and mating behaviour

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Abstract. The brine shrimp *Artemia* is an inhabitant of hypersaline environments and has been used to study primary reproductive traits and mating behaviour together with the degrees of divergence among populations. Intrapopulation experimental crosses and cross-fertility tests were evaluated in four populations from coastal and inland environments of South India, and in a reference sample of *A. franciscana* (San Francisco Bay -SFB, U.S.A). Crosses were fertile with the production of live nauplii and cysts. Significant variations were observed among the populations studied in fecundity (e.g. total offspring, brood size). A reproductive switch from ovoviviparity to oviparity was observed, especially in crosses between inland populations with the reference strain. Differences between the South Indian samples and SFB were significant- for the total number of nauplii, percentage of encysted brood and broods per female. A peculiar pattern of male-male mating was observed, wherein the ratio of males was higher than females. About 70% of the orientation movements of the males were directed towards other males.

Keywords: *Artemia*, cross fertility tests, mating behaviour, reproductive isolation.

Introduction

In South India, *Artemia* populations have been extensively characterized, but difficulties arise because of the ongoing description of newly discovered populations that have been introduced either for economic reasons or by natural dispersal. New *Artemia* locations have been reported in South India, and some of these populations have been identified as *A. franciscana.* Hence, populations can be quite heterogeneous genetically, some being even at the limit to be considered conspecific to *A. franciscana,* and causing serious identification problems if reliable traits are not used (Gajardo et al. 1999). As part of a broader characterization work of South Indian populations, this study examines the levels of reproductive isolation in *Artemia* populations in South India from San Francisco Bay (usually imported and used for mass culture of *Artemia* in South India).

Artemia tends to allocate maximum energy gamete and offspring production. The quality or type of zygote is significant for the survival of the brine shrimp population since two reproductive modes exist, and all strains combine both types with the ratio varying widely among them (Browne 1980, Gajardo & Beardmore 1989). Reproduction is either by ovoviviparity (when free swimming nauplii are released from the uterus), or by oviparity (when the embryos are provided with the thick shell from the shell gland of the female and enter a state of dormancy before being deposited). The processes leading from oviparity to ovoviviparity in *Artemia* are different, since it appears that embryos are always provided with a thick shell. Under some conditions they hatch from the shell within the ovisac, while under other conditions, development is arrested and cysts are laid. While this the general pattern, details such as the number of generations, the nature of relationship between oviparity, ovoviviparity, fecundity, sex ratios, the sort of environmental cues needed to provoke the protection of cysts and salinities at which cyst hatch, seem to differ between populations (Browne et al. 1984).

Natural selection provides heritable variants and differences between populations from different localities or interpreted as the result of adaptation to different environmental conditions. Reproductive isolation is also a key factor in the definition of species according to the biological species concept (Gajardo et al. 2001). Hence the process of speciation is considered equivalent to the development of barriers to crossing and, as a corollary of the allopatric mode, geographical or physical barriers between conspecific populations must exist of reproductive isolation to evolve as a by-product of the genetic differences.

In bisexual *Artemia,* laboratory cross-fertility tests have been the basis for the diagnosis of sibling species, and are considered the most appropriate criterion predicting the evolution of the genus. However, the rate of dissemination of cysts into new environments must be affecting the gene pool of many endemic populations (Gajardo et al. 1999), and so this criterion could be inadequate if there is no barrier to introgression (Browne & Bowen 1991). Information on mating behaviour in Anostraca is available for only 10 of the more than 200 described species. The observations on mating behaviour in anostracans have been summarized by Baird (1950). Mathias (1937) reviewed the subject and discussed the duration of amplexus, copulation time and mating posture. Experimental approaches on mating behaviour studies have also been attempted (Pearse 1913, Wiman 1979, 1981, Belk 1984). Cyst deposition behavior and the functional morphology of the brood pouch of *Streptocephalus torvicornis* has been studied Kraur et al. (2004). Rogers (2002) has studied the amplexial morphology of representative species from anostracan families. Females in the genus *Parartemia* and the families Chirocephalidae and Linderiellidae have been found to have morphological characters within their amplexial region that complement the ornamentation of the male's second antennae, creating a "lock and key" fit unique to each species. Recently, Benvenuto et al. (2009) has studied the mate guarding behavior and the community ecology of the clam shrimp, *Limnadia badia*.

Based on the available information it is generally assumed that male anostracans continually prowl for females. Males of *Artemia* and the unrelated *Artemioposis stefanssoni* seem to spend more time in amplexus and less in searching (Johansen 1921). Relying on visual cues, male anostracans orient toward any promising object. Males will attempt to clasp and mate with females that carry fertilized, shelled eggs and even females of other species. Anostracan females exercise choice of mates by not accepting every male that clasps them, even when it is in a receptive condition. A female is considered in receptive condition when she has mature, unfertilized eggs in the lateral pouches of the oviducts and an empty ovisac (Belk 1984).

The general mating pattern is the one in which males avoid aggression and simply search for receptive females. According to Moore and Ogren (1962), Wiman (1981) and Belk (1991), anostracan mating behavior follows a set pattern: detection, orientation, station taking, amplexus, intromission, copulation and disengagement. Females acquire resources on their own because their mates provide nothing, and thus females are free to choose among all available males on the basis of phenotype differences reflecting relative genetic quality (Brendonck & Belk 1997).

The concept of scramble-competition phylogeny helps us to understand mating behaviour in the context of anostracan ecology and in the context of how individual males behave in an effort to leave more offspring than their rivals. Moreover, knowledge of the reproductive structures is an essential criterion to understand the anostracan mating behaviour (Brendonck & Belk 1997, Rogers 2002). The morphology of male anostracan antennae takes on great variety in the form of outgrowths ranging from surface texturing, spines, knobs and other processes to the elaborate medial processes, antennal appendages and frontal appendages. These undoubtedly function in providing tactile cues during premating courtship when a male clasps a female and brings them into intimate contact with her body (Belk 1984, Gajarado et al. 2001).

Sexual differences in the spatio-temporal distribution and mortality of organisms due to natural and artificial factors often lead to local variation in field sex ratios. The sex ratio is directly related to the operational sex ratio, which is defined as the ratio of fertilizable females to sexually active males at any given time (Wiman 1978), and can affect the mating system of an organism.

In the present study, the primary reproductive traits of *Artemia* populations of different origins of South India were compared with the reference strain of San Francisco Bay along with cross-fertility tests. Furthermore, I examined the possible impact of sex ratio on mating behaviour in *Artemia* spp. collected from Kelambakkam, Chennai, Tamil Nadu.

Material and Methods

The samples of *Artemia* populations used in the present study are indicated in Table. 1. South Indian populations correspond to laboratory reared brine shrimps descended from wild animals collected in coastal ponds (Kelambakkam (KBM) and Tuticorin (TUT)) and inland lakes (Puthalam (PUT) and Thamaraikulam (TKM)) and maintained in the laboratory for 2-3 months (1-3 generations). The salinity of 60 ppt, on which *Artemia* cultures were reared before the breeding tests, was selected for convenience recognizing that *Artemia* strains show high survival over a wide range of salinities (35-110 ppt) (Vanhaecke et al. 1984).

Coastal ponds remain ephemeral at Tuticorin, experiencing the effects of rain- which can lower salinity significantly. The inland populations exhibit extreme ecological characteristics and are often subject to significant fluctuations in ionic composition, salinity and temperature. Samples from San Francisco Bay (SFB: *A. franciscana* Kellogg, 1906) were commercially procured and were hatched following standard protocols (Sorgeloos et al. 1986).

Intrapopulation and cross fertility tests

Intrapopulation reproductive performances as well as interpopulation crosses, or cross fertility tests, were carried out at 60±2 ppt (artificial sea water), from single-pair reciprocal crosses of adult *Artemia* following the standard laboratory procedure described by Gajardo et al. (1998), which enables the evaluation of prime reproductive characteristics in a number of individual crosses with a fairly good repeatability. Crosses were considered fertile when live nauplii and cysts were produced. For experimental crosses, freshly hatched nauplii were cultured in three tanks, and before sexual maturity, randomly selected pairs were transferred into separate vials containing 50 ml of artificial sea water (Kinne, 1971). On alternate days and just before feeding (1000 cells of *Chlorella vulgaris* Beijerinck, 1890 / vial per day), ovoviviparous offsprings were counted and transferred into separate bottles containing 200 ml of brine, where they were maintained until maturity. Oviparous offsprings (cysts) were filtered and counted (Gajardo & Beardmore 1989). Observations lasted for 50 days, a time considered sufficient for most of the reproductive spawns to have elapsed (Gajardo & Beardmore 1989). Cross-fertility experiments were repeated for 10 pairs of each population with San Francisco population.

Mating behaviour

Artemia collected from Kelambakkam (KBM) were acclimatized in the laboratory for two days at a salinity of 60±2 ppt. The observation containers were 100 x 50 mm diameter flat-bottomed Pyrex dishes filled with 10 mm of the top with water and the males and females were placed in these dishes at different sex ratios. *Artemia* were fed with *Chlorella vulgaris* and water containing fecal and excess algal clumps was removed and replaced by an equal amount of fresh brine.

Three experimental setups with different combinations of male and female sex ratios were maintained for the study of mating behaviour. The total number of brine shrimps was restricted to 20 in each container, containing filtered seawater. For all three experiments, triplicates of samples were maintained. In Experiment I, ten receptive females and ten males were introduced into each dish. Experiment II had 15 receptive females and 5 males and Experiment III was carried out with 5 receptive females and 15 males in each dish. The animals were checked for mating at every ½ hour interval for three day and night. All observations were conducted at a temperature of 32±2°C and the alga *Chlorella vulgaris* used as the food.

Statistical analysis

Overall reproductive differences and variations among populations from different origin were performed by One and Two-way ANOVA using SPSS 10 for Windows (SPSS Inc., Chicago, IL, USA), and data are given as arithmetic means ± standard deviations (Sokal and Rohlf, 1981). A probability value of 0.05 or less of the statistical tests between the control and experimental values was considered significant.

Results

Intrapopulation variation

Table 2 shows the reproductive performance and significance of mean differences (ANOVA), for 6 reproductive traits in the *Artemia* populations studied. ANOVA showed overall significant differences among populations for particular traits such as total number of cysts per female ($P<0.01$) and number of broods per female ($P<0.05$). South Indian populations- exhibited both the largest (561, TUT) and smallest (387, SFB) total offspring, whereas the percentage of encysted offspring varied widely among the strains from a minimum of 23% (SFB) to a maximum of 59% (TUT). All the populations were efficient in switching, in a significant way, their reproductive mode to nauplii with low cyst production ($P<0.05$). The number of broods per female varied between 3.9 (SFB) to 6.7 (TUT), whilst the interbreed interval (days between broods) was about 3.1 – 3.7 days.

Cross fertility tests with reference sample

Reproductive performances and encystment ratios were evaluated for cross fertility tests with reference sample of *A. franciscana* (SFB) and other populations viz., Puthalam (PUT), Thamaraikulam (TKM), Tuticorin (TUT) and Kelambakkam (KBM). All the tests carried out were fertilty and fecundity (offspring per female), offspring quality (cysts or nauplii), and percent offspring encysted (Table 3). Overall, differences between the South Indian samples and SFB were significant-, for total number of nauplii ($P<0.05$), percentage of encysted brood ($P<0.001$) and broods per female ($P<0.05$). For total offspring per female, crosses with SFB varied from 421 (TKM) to 738 (PUT).

Mating behaviour

In the experiments conducted, interesting observations were recorded on the mating behaviour of *Artemia* (Table 4). In experiment I,

Table 1. *Artemia* populations used in this study

Country	Locality	Abbreviations	Geographical Co-ordinates	Type of habitat
South India	Puthalam	PUT	08° 06′ 27 N 77° 27′ 59 E	Inland
	Thamaraikulam	TKM	08° 07′ 04 N 77° 29′ 17 E	Inland
	Tuticorin	TUT	08 ° 47′ 56 N 78° 08′ 38 E	Coastal
	Kelambakkam	KLB	12° 48′ 00 N 80° 13′ 47 E	Coastal
U.S.A.	San Francisco Bay	SFB	37° 42′ 55 N 122° 16′ 41 W	Coastal

Table 2. Mean reproductive performance (X ± SD) in six characteristics of five *Artemia* populations - San Francisco Bay (SFB), Puthalam (PUT), Thamaraikulam (TKM), Tuticorin (TUT) and Kelambakkam (KBM) on 50 days of observation.

Reproductive traits	Populations				
	SFB	PUT	TKM	TUT	KBM
Total offspring/female (no. of pairs in parenthesis)	387±84 (8)	432±109 (11)	394±54 (8)	561±178 (9)	502±197 (6)
Cysts/female**	110±24	173±55	137±14	285±64	243±41
Nauplii/female	279±42	309±27	291±32	384±71	325±42
Offspring encysted(%)	23±7	46±12	37±4	59±11	52±14
Broods/female*	3.9±0.4	5.2±0.7	4.3±0.6	6.7±0.7	6.1±0.3
Days between broods	3.1±0.2	3.6±0.4	3.2±0.7	3.3±0.4	3.7±0.5

Statistical significance among populations P<0.05*, P<0.01**

Table 3. Results of intraspecific and interspecific crosses between the *Artemia* population from San Francisco Bay (SFB) and reference populations from Puthalam (PUT), Thamaraikulam (TKM), Tuticorin (TUT) and Kelambakkam (KBM) (mean values/female) over 50 days of observation.

Reproductive traits (SFB)	Populations			
	PUT	TKM	TUT	KBM
Total offspring/female	738 ± 209	421 ±75	502 ± 179	468 ± 96
Cysts/female	521 ±175	257 ± 67	198 ±19	168 ± 42
Nauplii/female*	261 ± 72	193 ± 51	156 ± 29	142 ± 21
Offspring encysted(%)**	67 ±11	46 ± 9	28 ± 11	32 ± 7
Broods/female*	7.6 ± 1.4	5.7 ± 0.8	4.7 ± 0.9	3.5 ± 0.6
Days between broods	5.8 ± 0.3	4.0 ± 0.7	3.0 ± 0.8	2.7 ± 0.4

Ẍ ±S.D. of ten observations
Statistical significance among populations P<0.05*, P<0.001**

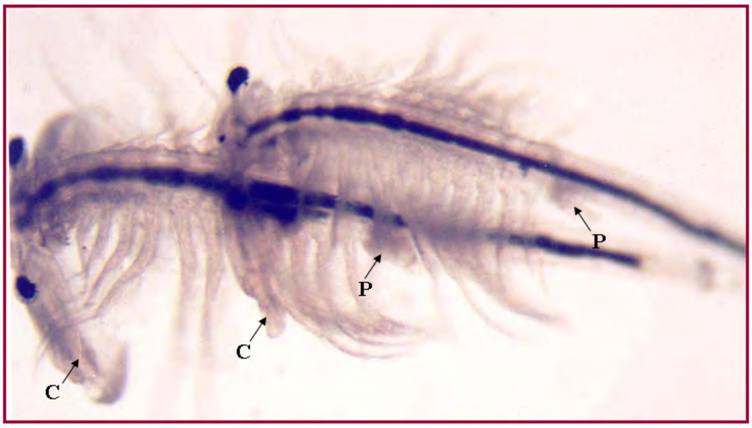

Figure 1. Photograph showing male-male mating behaviour of *Artemia* with the 2nd male clinging on to the 1st male with the help of its claspers (C- Claspers, P- Penis).

Table 4. Effect of sex ratio on the mating pattern of *Artemia* population from Kelambakkam (KBM)

Expt.	Female:Male ratio	Mating behaviour					
		Female-male riding			Male-male riding		
		24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
I	1:1	2 ± 0	-	-	-	-	-
II	15:5	5 ± 1	8 ± 2	3 ± 1	1 ± 0	-	-
III	5:15	3 ± 0	2 ± 0	3 ± 0	6 ± 1	5 ± 0	6 ± 2

Ẍ ± S.D of six observations

with equal numbers of female and male *Artemia*, only two male and female ridings were observed. The results were further confirmed by the presence of unfertilized eggs in the lateral pouches of the females. In contrast, experiment II, with more females present than males, all 15 females mated. This was supported by evidence that the movement of eggs from the lateral pouches into the ovisacs and that these eggs were shelled two days later.

A peculiar pattern of male-male mating was observed in experiment III, wherein the ratio of males was higher than females. About 70% of the orientation movements of the males were directed towards other males. A male precisely clasps the thoracic region of other male in a similar pattern of riding with a female (Fig. 1). The maximum number of male and male pairs (6 pairs) were observed during the early hours of observation and remained segregated from each other only after 3-4 hrs.

Discussion

Understanding the evolutionary potential of *Artemia* populations exposed to new or different seasonal habitats could be done by studying their reproductive performances. Adaptive patterns among the *Artemia* populations from different origins of the present study are well reflected on comparing their reproductive characteristics.

Intraspecific comparisons

The quality of cysts and nauplii is an important reproductive strategy ensuring survival in population exposed to stress. Cyst production also plays a significant role in the geographical dispersion of any species as cysts are known to be transported by wind or birds (Vanhaecke et al. 1987). By comparing reproductive efficiency and quality of *Artemia* populations from different origins, this work probably reflects the adaptation pattern resulting as a compromise between natural populations and their local habitats. Indeed, since the introduction of *A. franciscana* in South India for commercial purposes, rapid evolutionary changes seem to have occurred, paralleling some phenotypic differences. In the present study, South Indian *Artemia* populations showed important variations in total broods per female and % offspring encysted. At least a fraction of the variability is heritable and so populations studied reflect the trend towards maintenance of high levels of genetic variability in their gene pools and great interpopulation genetic heterogeneity (Abreu-Grobois 1987).

Although the factors controlling the mode of reproduction are not fully understood, oviparity in *Artemia* is generally considered to be induced by environmental stress (Lavens & Sorgeloos 1984). Salinity shocks have been found effective in switching the population towards cyst production (Sorgeloos 1989). In addition, low oxygen and dissolved oxygen concentration reportedly induce oviparous reproduction in *Artemia*. This condition thus stimulates the synthesis of haemoglobin and excretion by brown shell gland, haematin, which is the main constituent of the cyst shell of *Artemia* (Lavens & Sorgeloos 1984). This study has afforded an opportunity to evaluate the prime reproductive characteristics in individual crosses. In this way, a whole range of genetic types could be monitored under environmental conditions. Recently, Rafael et al. (2009) have compared two pure zygogenetic strains of *A. franciscana* from San Francisco Bay and an *Artemia sp.* from a natural population in Mexico. They has been observed that a significantly lower proportion of males or a significantly higher proportion of females can preliminarily detect the presence of parthenogenetic individuals upon incubation below 33°C.

Cross-fertility tests

Cross fertility tests have traditionally been the basis for the diagnosis of *Artemia* species (Browne & Bowen 1991). Standardized laboratory studies with *A. franciscana* have shown that many morphologically or genetically divergent allopatric populations exhibit partial or no reproduction (Pilla & Beardmore 1994). Also fertile F1 and viable F2 adult have been obtained in reciprocal crosses between *A. monica* and *A. franciscana* (Bowen et al. 1985). The present study has made possible the evaluation of variability and divergence of populations for complex traits such as encystment, which is known to be affected by hybridization and heterozygosity in *Artemia* (Gajardo & Beardmore 1989, Browne & Bowen 1991). Hence, when the cyst production is high in any production, the more heterozygous females may be more resistant to adversity than less heterozygous ones and more able to produce cysts for longer and with a wider range of genotypes. In our present study, encystment-/ovoviviparity ratios increased in cross-fertility tests, between South Indian inland and SFB populations. Conversely, there was a decrease in all performances (including the percentage offspring encysted) after hybridization in the coastal populations. Several authors have postulated that the frequency of oviparity in *Artemia* is not only correlated with environmental stress but is also influenced by geographical strain of *Artemia* which has been used for inoculation (Browne et al. 1984). Dependence of oviparity is commonly seen in populations experiencing a seasonal cycle of either temperature of salinity of living habitats. Saavedra and Amat (2005) have performed crosses of two populations of *A. franciscana* differ in the number of cysts they produce which showed a maternal effect of encysted broods. An evolutionary consequence of shunting offspring into a dormant encysted state is to ensure the survival of the species once favourable conditions return, and this might occur in geographical and temporal conditions different from those experienced by the parents. This probably explains why there is no statistical difference in reproductive strategy when coastal and inland populations were compared in the present study.

Gajardo et al. (1999) also obtained viable crosses with the production of cysts and nauplii. The possibility to perform cross-breeding under laboratory conditions between geographically-separate populations (as seen in the present study)-, could then be interpreted as an indication that the development of barriers to gene exchange has not yet been perfected (Pilla & Beardmore 1994, Gajardo et al. 1999).

Mating behaviour

Bisexual *Artemia* present some interesting differences in anostracan reproductive biology. The comparative value of understanding these differences, although with the economic importance of *Artemia*, suggests that it would be worthwhile to carefully study mating behaviour in *Artemia*. The first contact between sexes seems to be realized by the male antennae and antennules touching the female genital region. Intromission only occurs when both sexes have gone through all courtship phases successfully (Mathias 1937, Moore & Ogren 1962, Wiman 1981, Freiner & Gruttner 1984, Belk 1991). The reliance of anostracan males on cues that are less significant for detection, orientation, station taking and clasping, most likely derives from the nature of the competitive scramble for the rare receptive female.

Results from the present study amply demonstrate the importance of sex ratio in the mating behaviour of *Artemia.* The least mating observed was during experiment I with a sex ratio of 1:1, and clearly indicates the need for competitive courtship. Belk (1984) has also conducted similar experiments with *Eubranchipus serratus* Forbes, 1876 and has enumerated the importance of sex ratio in mating. A higher ratio of females than males may have paved the way for competition among the females in experiment II. Furthermore in experiment III, competition prevailed among males to mate the available females. Wiman (1978) hypothesized that a sexually receptive anostracan female may increase her chances of encountering a male by becoming more active.

Wiman (1981) reported that the anostracan male is in a competitive environment in which the few seconds required in detection, orientation, station taking and clasping attempts are of little consequence as compared to the risk of missing a chance to clasp a receptive female. Belk (1984) reported that due to the low intensity of male-male competition, competitively inferior males may fertilize a higher percentage of the females under female- biased conditions than when the sex ratio is even or male-biased. On the other hand, many receptive females might not be able to be mated and/or fertilize their eggs in extremely female-biased populations because of the lack of male sperm or energy limitations.

The occurrence of male-male mating behavior in experiment III, explains the inadequate supply of females and low chances for clasping females. The males, not withstanding consistent rejection by females, either continue to attempt mating females or simply clasp the other males. Belk (1984) has reported that males will mate or clasp females in an attempt to mate repeatedly, however, receptive females typically reject a number of mating attempts before finally accepting a mate. Unusual pairing between brine shrimps in crowded conditions existing in culture jars has been recorded. Rarely, a male has been found to clasp the abdomen of male, which already has an appropriate grip upon the ovisac of female, producing a triple set of *Artemia*. Further, triple co-ordination as well as between male-male pairs argues strongly against the existence of any special structure in the female, which could respond to some signal emanating from the male. It has been postulated by Brendonck and Belk (1997) that the specific mate recognition in anostracans occurs early in the courtship sequence and is most likely based on differences in precopulatory signals (e.g. swimming behavior and possibly chemical cues), and in noncopulatory contact organs such as male antennal and telsonic structures, and body spinulation, all of which are useful taxonomic characters at the species level.

The importance of sex ratio in the study of reproductive patterns of Anostraca has been documented well. Studies of Belk (1984) have also emphasized the significance of sex ratio in fairy shrimps. Pearse (1913) pointed out, male *Artemia* are continually ready to mate and will mate repeatedly. Females, on the other hand, are ready to mate only when they have fertilized eggs in their lateral oviductal pouches. The data of Belk (1984) suggest that even females in receptive condition reject a percentage of mating attempts and are about 4 times likely to reject males lacking antennal appendages than intact males.

Finally, the reproductive behaviour observed in *Artemia* populations studied may be a response to the very broad range of ecological variables typically found in *Artemia* habitats.

Moreover, the influence of sex ratio on mating behaviour of *Artemia* would pave way in understanding innate behaviour of reproductive efficacy of the brine shrimp *Artemia*.

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Submitted: 24 February 2008
/ Accepted: 29 July 2010