Selective Advantage for III^M Males over Y^M Males in Cage Competition, Mating Competition, and Pupal Emergence in *Musca domestica* L. (Diptera: Muscidae)

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ABSTRACT In the house fly, *Musca domestica* L. (Diptera: Muscidae), sex is usually determined by a dominant factor, M, located on the Y chromosome. However, there are autosomal male (A^M) populations in which the M factor is located on one or more of the five autosomes (I–V), most commonly on the third chromosome. Herein we report the use of isogenic strains to determine the relative fitness of Y^M versus III^M males in three different experiments. First, cages were started with 50% Y^M and 50% III^M males, and the frequencies of Y^M and III^M males were evaluated across generations. Second, mating competition studies were preformed with these isogenic strains. Third, the relative emergence rates of III^M versus Y^M male pupae held at three temperatures for 3 d were examined. All three studies indicate that III^M males have a greater fitness than Y^M males. In the cage competition studies, >90% of the males were III^M after seven generations. III^M males were more likely to mate than Y^M males, and a higher percent of III^M males emerged after being held as pupae at 4, 16, or 28°C for 3 d. The implications of these studies to the distribution of III^M and Y^M males in field populations are discussed.

KEY WORDS M factor, sex determination, isogenic strain, fitness

Male sex determination in the house fly can be accomplished by linkage of the M factor to the sex chromosome (Y) or to an autosome (I–V). III^M males seem to be the most frequently occurring, being found in a number of countries including the United States (Hiroyoshi 1964, McDonald et al. 1975, Shono and Scott 2003, Hamm et al. 2005), Japan (Hiroyoshi 1964, Hiroyoshi et al. 1982, Tomita and Wada 1989, Shono and Scott 1990), Turkey (Cakir 1999), Italy (Franco et al. 1982), the British Isles (Denholm et al. 1985), South Africa (Denholm et al. 1990), and Australia (Hamm and Scott 2009). Clines (in the relative frequency of A^M and Y^M) have been reported in the United States, Japan, and Europe. In the United States, flies collected from Maine were all Y^M, whereas in Florida, 100% of the males were III^M. Flies collected in 1973 from Florida were also III^M (McDonald et al. 1975), leading to the idea that there is some selective advantage for autosomal males in Florida because of the stability of III^M males for the past 30 yr. North Carolina and New York were intermediates along this cline (Hamm et al. 2005). A significant north–south latitudinal cline was also found in Japan, with a higher percentage of A^M in the south (Tomita and Wada 1989). Northern populations were mostly Y^M males. Franco et al. (1982) reported an altitudinal and latitudinal cline was present from locations in Denmark and Iceland in the north to Sicily in the south. Populations were mostly A^M south of the 44th parallel and/or below 100 m above sea level. Autosomal males decreased in frequency as the altitude increased. Recently, the cline in Europe was re-examined, and the relative distribution of A^M and Y^M males was relatively unchanged, and populations with both A^M and Y^M males were found to be stable (Kozielska et al. 2008).

The presence of similar clines in the United States, Japan, and Europe leads to a number of questions, such as what selective advantages are present for the various linkages of M in different environments? The first attempt to identify an environmental condition that might influence the frequency of A^M versus Y^M males was conducted by Cakir and Kence (1999). The Trabzon and Izmit strains from Turkey were 100% XX males, with an unknown linkage of M. The Polatli population was XY with 3% XX males. Two sets of cages were started with 30 pairs of virgin adults at ratios of 1:9, 5:5, and 9:1 using the Trabzon: Polatli and Izmit: Polatli strains. One set was kept at 25 ± 3°C and the other at 35 ± 3°C. At each generation, males were cytologically examined. The authors claimed there was an increase in the frequency of XX males in all populations, except the five Izmit: five Polatli mixture at 25°C, which had no change over six generations (Cakir and Kence 1999). However, no statistical tests were run to determine whether differences were significant from one generation to the next. Table 1 from

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that study seems to have data that contradicts the claim of an XX advantage, and the strains used did not share a common genetic background (Cakir and Kence 1999). Thus, the conclusion that there is a fitness advantage for autosomal males in competition with Y males is difficult to judge.

This study used isogenic strains to determine the relative fitness of Y^M versus III^M males in three experiments. In one study, cages were started with 50% Y^M and 50% III^M males and kept at 28°C with a 12:12-h light:dark photoperiod. Frequencies of each male type were evaluated across generations. Mating competition studies were also performed with these isogenic strains. In addition, the relative emergence rates of III^M versus Y^M male pupae were examined after being held for three days at one of three temperatures.

Materials and Methods

Isolation of Near Isogenic III^M and Y^M Strains. The Cornell-Susceptible (CS) strain has the M factor on the third chromosome (III^M) (Hamm et al. 2005). The ME 2002 strain (collected from a dairy in Androscoggin County, ME) is entirely Y^M. These two strains were used to construct a Y^M strain (CSME) that was near isogenic to CS. Unmated females (≤8 h after emergence) from the CS strain were crossed to ME 2002 males. F_1 males were backcrossed to unmated CS females. This process continued for 12 generations. More than 340 females were mated with >100 males each generation. All flies and larvae were kept at 28°C with a 12:12-h light:dark photoperiod. House fly larvae were reared on medium made with 1.8 liters of water, 500 g calf manna (Manna Pro, St. Louis, MO), 120 g bird and reptile litter wood chips (Northeastern Products, Warrensburg, NY), 60 g dried active baker's yeast (MP Biomedicals, Solon, OH), and 1,210 g wheat bran (Cargill Animal Nutrition, Minneapolis, MN). Adult flies were kept in mesh cages (35.6 by 25.4 by 26.7 cm) provided with a 1:1 mix of sugar and powdered milk and water *ad libitum.*

To confirm the CSME strain was Y^M, a backcross experiment was carried out as previously described (Hamm et al. 2005) using a strain (aabys) with visible recessive markers *ali-curve*, *aristapedia*, *brown body*, *yellow eyes*, and *snipped wings* on autosomes I–V, respectively. Male CSME flies were individually crossed to three to six unmated aabys females. Thirty males were crossed for this strain and resulted in 17 crosses with flies for the subsequent backcross analysis. Emerging F_1 males and females were counted. Three F_1 males from each original male were individually used in a backcross with three to six aabys females. The emerging backcross (BC) individuals were phenotyped according to sex and body color marker (marker on chromosome III). All CSME males were Y^M (i.e., all males lacked an association with any visible recessive autosomal marker). To confirm that insecticide-resistance alleles (often associated with a fitness disadvantage) found in the ME strain were not present in CSME, partial *Ace*, *CYP6D1*, and *Vssc* alleles were sequenced from at least 20 individual CSME flies. The CSME strain contained only *Ace* and *CYP6D1* insecticide-susceptible alleles (data not shown). The *Ace* allele is located on chromosome 2 and the *CYP6D1* allele is on chromosome 1. However, the CSME strain contained a low frequency of the *kdr* allele (mutation in the *Vssc* gene [on chromosome 3] conferring resistance to DDT and pyrethroids).

Thirty single pair crosses from the CSME strain were set up to create the IsoCS strain. Parents from single pair crosses that produced high numbers of larvae were genotyped through sequencing of polymerase chain reaction (PCR) products for *Vssc* as previously described (Rinkevich et al. 2006). Offspring from four single pair crosses that had homozygous susceptible genotypes for both parents were combined to create the IsoCS strain. Thus, the IsoCS strain is homozygous susceptible for *Vssc*, *Ace*, and *CYP6D1*, has Y^M sex determination, and is isogenic to CS. The CS strain is homozygous susceptible for *Vssc*, *Ace*, and *CYP6D1* has III^M sex determination.

Random Amplified Polymorphic DNA. Random amplified polymorphic DNAs (RAPDs) were carried out to find a marker to differentiate between the Y^M and III^M males. We were able to find male-specific markers for the Y^M and III^M strains (see below).

Flies were homogenized in 2 ml extraction buffer (100 mM Tris, 1% SDS, 100 mM EDTA, and 0.2 mg/ml proteinase K). The homogenate was transferred to a 2-ml tube and incubated at 65°C for 90 min and then centrifuged at 13,000*g* for 10 min at 4°C. The supernatant was transferred to a 15-ml tube, 2 ml of phenyl/chloroform/isoamyl alcohol was added, and the sample was mixed by hand for 10 min. Samples were centrifuged for 5 min at 4,000*g.* The aqueous solution was transferred to a new tube, and 2 ml of chloroform was added, shaken for 10 min, and centrifuged as above. For every 1 ml of aqueous solution, 2.5 ml of 100% ethanol was added in a new tube to precipitate DNA. The solution was centrifuged briefly, and the supernatant was discarded. The DNA pellet was washed with 2 ml of 70% ethanol. The tube was centrifuged at 13,000*g* for 5 min, and the supernatant was discarded. The pellet was air-dried for 10 min and dissolved in EB Buffer (10 mM Tris-HCl, pH 8.5) at 20 mg/ml, and genomic DNA (gDNA) was measured using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Screening of RAPDs was done by PCR in a 25-μl reaction containing 19 μl ddH_2O, 2.5 μl 10× Advantage 2 PCR thermobuffer, 0.5 μl 10 mM dNTPs, 0.5 μl Advantage two polymerase mix (Clontech Laboratories, Mountain View, CA), 1 μl of 20 ng/ul gDNA, and 1.5 μl of primer (25 μM). We screened arbitrary 10-mer primers including the following: OPA12, TCGGCGATAG; OPD 15, CATCCGTGCT; OPG01, CTACGGAGGA (Operon Biotechnologies, Huntsville, AL). The reaction was carried out in a Hybaid PCR Express thermal cycler (Teddington, Middlesex, United Kingdom) under the following conditions: 95°C for 2 min, followed by 47 cycles of PCR (95°C for 0.5 min, 37°C for 1 min, and 72°C for 2 min), and a final extension cycle of 72°C for 5 min. PCR

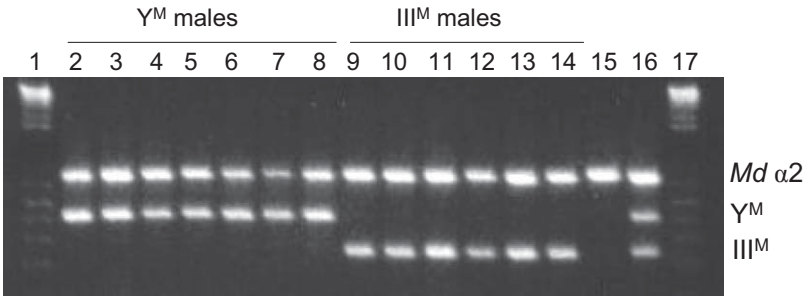

Fig. 1. PCR products for Y^M (lanes 2–8) and III^M (lanes 9–14) specific markers. The nicotinic acetylcholine subunit, *Mdα2*, was used as a positive control for each DNA sample. A mix of Y^M and III^M males provided a positive control, and a female was used as a negative control for each gel. Lanes 1 and 17 are size markers (1-kb ladder), lane 15 is a female, and lane 16 contains the PCR product from a 1:1 mixture of genomic DNA from a Y^M and III^M male.

products were analyzed by electrophoresis on 1.8% agarose gels stained with ethidium bromide. A 1-kb plus DNA ladder (Invitrogen, Carlsbad, CA) was run on each gel.

Candidate bands were excised and purified from the gel and cloned into pCR2.1-TOPO vector using the Topo TA Cloning Kit (Invitrogen). The 6-μl reactions contained 3 μl sterile water, 1 μl PCR product, 1 μl salt solution, and 1 μl of pCR2.1-TOPO vector. The solution was mixed and incubated for 8 min at room temperature, after which it was placed on ice. Top 10 competent cells were transformed by adding 2 μl of the TOPO cloning reaction to a vial of Top 10 cells. The reaction was incubated on ice for 20 min. Cells experienced a heat shock at 42°C for 30 s and were immediately returned to ice. S.O.C. medium (2% trypyone, 0.5% yeast extract, 10 mM NaCl, 2.5% KCl, 10% $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose; 250 μl) at room temperature was added. The reaction was shaken at 37°C for 1 h at 200 rpm. LB plates containing ampicillin and X-gal were spread with 5, 15, or 50 μl of the bacteria suspension. *E. coli* were incubated at 37°C for 1 h.

Single white colonies from each transformation were individually added to LB medium containing 100 μg/ml ampicillin and incubated at 37°C overnight. Plasmids were isolated using the QIAprep Spin Miniprep Kit according to the manufacturer's suggestions (Qiagen, Valencia, CA). The insert was sequenced with M13 forward primer at Cornell's Biotechnology Resource Center. Sequence of the inserts was used to design primers to specifically amplify III^M or Y^M PCR products. The accession numbers for the III^M and Y^M male specific sequences are EU687740 and EU687741, respectively.

gDNA Extraction for Genotyping. Individual flies were frozen at −80°C. Whole male flies were used, whereas females had their abdomens removed. Animals were completely pulverized with a disposable pestle (Kontes Glassware, Vineland, NJ) in 200 μl of buffer (100 mM Tris-HCl, pH 7.5, 100 mM EDTA, 100 mM NaCl, 0.5% SDS). An additional 200 μl of buffer was added before incubation at 65°C for 30 min. After incubation, 800 μl of LiCl/KAc (1 part 5 M KAc: 2.5 parts 6 M LiCl) was added, mixed, and left on ice for at least 10 min. The samples were spun at 14,100*g* for 15 min at 25°C, 1 ml of the supernatant was transferred to a new tube, and 520 μl of isopropanol was added and mixed. The samples were spun again at 14,100*g* for 15 min at room temperature. Liquid was aspirated away and discarded. Pellets were washed with 180 μl of 70% ethanol and spun at 14,100*g* for 30 s. The final pellet was allowed to air dry for at least 30 min and was resuspended in 150 μl of EB buffer for males or 75 μl EB for females.

PCR Assay for III^M and Y^M. Primers were developed based on the RAPDs results to distinguish between III^M and Y^M males. Primers used (5′ to 3)′ were as follows: A12CMF1 (TCG GCG ATA GGG AGA AAA AGT AGA), A12CMR1 (TCG GCG ATA GCA AAG TCG GTA ATG), F15CSF1 (CCA GTA CTC CAC AAC AAA GAT ATT T), F15CSR1 (CCA GTA CTC CTT TAA AAA ATA GCT C), GM2IIIF1 (AAG GAT CAG ATT CTA ACC ACA AAC GTG T), and GM2VIR2 (ACA TGC CGA CGG TGG GAA TGA TCA G). A12CMF1 and A12CMR1 were used for the Y^M-specific PCR product, F15CSF1 and F15CSR1 were used for the III^M-specific product, and GM2IIIF1 and GM2VIR2 are primers for *Mdα2* (Gao et al. 2007), which was used as a positive control (to ensure amplification) of each sample. PCR was done in a 25-μl reaction containing 5.5 μl ddH_2O, 6 μl of primer mix, 1 μl gDNA, and 12.5 μl of 2× ReddyMix (ABgene House, Surrey, United Kingdom). The reaction was carried out in a Bio-Rad iCycler thermal cycler (Bio-Rad, Hercules, CA) under the following conditions: 95°C for 2 min, followed by 32 cycles of PCR (95°C for 0.5 min, 52°C for 0.5 min, and 72°C for 1 min), and a final extension cycle of 72°C for 5 min and a hold at 4°C until used or frozen. PCR products were analyzed by electrophoresis on 1.8% agarose gels stained with ethidium bromide. A 1-kb plus DNA ladder (Invitrogen) was run on each gel. Parental CS and IsoCS males ($n = 55$ for each strain) and females ($n = 20$ for each strain) were used to verify the markers. All males had a Y^M or III^M band, and all females lacked both the Y^M and III^M bands (Fig. 1).

50:50 (Y^M:III^M) Population Cages. Four replicate cages each containing 300 unmated females, 150 Y^M unmated males, and 150 III^M unmated males were set

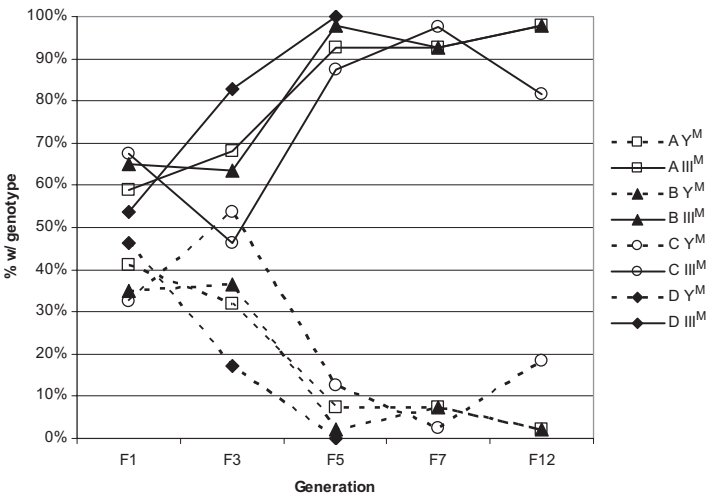

Fig. 2. Changes in the percentage of III^M and Y^M male house flies in four replicate cages (A–D) over 12 generations of random mating under standard laboratory conditions.

up. Cages A and B contained CS females, whereas cages C and D used IsoCS females. Females should be equivalent because these are isogenic strains. All parental flies were 2–3 h old when they were introduced to the cages. Eggs were collected at least 2 different d per generation from 3- to 5-d-old flies. Each subsequent generation was made with a minimum of 800 flies released into cages. Each generation, 200 male flies from each cage replicate were collected and frozen at −80°C for gDNA extraction and genotyping. At least 40 individual males were genotyped for III^M or Y^M from each generation tested (F1, F3, F5, F7, and F12). Given the equivalence of a haploid system (one Y chromosome) with male sex determination, we can apply a model for fitness that has been used for bacterial systems (Hartl and Clark 2007). Fitness was estimated with the equation $\log(p_t/q_t) = \log(p_o/q_o) + t\log(w)$, where p_t is the frequency of Y at generation t and $q_t = 1 - p_t$ is the frequency of III^M at generation t. The relative fitness of Y^M versus III^M is w. An estimate of w is calculated as $\log(p/q)$ for each generation and running linear regression of $\log(p/q)$ on generation t. The slope is $\log(w)$. Fitness is $w = e^{\log(w)}$.

Mating Competition. Males and females were sorted every 8 h and held in separate cages to ensure females were unmated. Male flies were marked with either yellow (114A; N.Y.C., Farmingdale, NY) or orange (112A; N.Y.C.) nail enamel to distinguish between Y^M and III^M males. Reciprocal colors were performed on flies during each experimental day and time. Three- to 5-d-old males were introduced into a 180-cm^2 (230 ml) glass jar containing a female of the same age. Flies were observed for up to 8 h for mating. A mating was scored if the male fly remained attached to the female for >1 min. The average mating speed for single pair crosses was found to be ≈30 min and a copulation time of over 1 h (Bryant 1980). A χ^2 test was performed to determine the significance of effects of male strain, female strain, and nail enamel color.

Temperature Effects on Emergence of Pupae. Synchronized egg batches were collected from CS and IsoCS colony cages and reared identically to be used in this study. Pupae from each colony were sorted during tanning. Cream color pupae were collected and left to tan at 28°C for 2 d. Pupae were randomly assigned a treatment condition of 4, 16, or 28°C. Pupae were counted and placed into wax cups with bridal veil covers. Two to three cups of 100 pupae from each strain were placed into a Lock & Lock Container (131 oz) with a poly chiffon fabric covered top containing 1,000 ml of water in the bottom for humidity. A platform was constructed so the wax cups did not contact the water. Pupae remained at this treatment temperature for 72–76 h and were placed at 28°C for emergence. Emerging flies were counted. Percent emergence of the total pupae that emerged as males for each temperature was arc sin-transformed, and Tukey's *t*-test was performed for all pairwise comparisons between temperatures and strains. Statistics were done using formulas entered into an Excel spreadsheet.

Results

50:50 (Y^M:III^M) Population Cages. Autosomal III^M males had a clear fitness advantage over Y^M males in all replicate cages (Fig. 2). Even in the first generation, III^M males were found at greater than the expected 50% frequency. Fixation for III^M males occurred by generation 5 in cage D. All cages contained >85% III^M males by generation 5. Cages A and B remained relatively constant in frequencies from generations 5 to 12, whereas cage C has some irregular shifts decreasing III^M males in generations 3 and 12. The relative fitness of Y^M males was calculated to be 0.47.

Mating Competition. In mating competition studies, a highly significant effect ($P < 0.0001$) of male type was found (Table 1). Autosomal III^M males (CS) were

Table 1. Mating competition between male CS (III^M) and IsoCS (Y^M) male house flies

Strain	Male		Total	Expected[a]	χ^2	P
	Yellow	Orange				
CS	131	144	275	201	27.24	<0.0001
IsoCS	71	56	127	201	27.24	<0.0001

Females from CS and IsoCS strains were pooled because they showed no effect on mating competition.

[a] Total no. expected if each male had an equal mating probability.

more effective at mating with females first, regardless of female strain. Relative to III^M males, the Y^M male mating success was 127/275 = 0.46. This is very close to the 0.47 relative fitness found in the cage experiment. The ability to find a mate is a clear fitness advantage because female house flies usually only mate once (Keiding 1986). This could explain the rapid shift seen in the cage studies. If III^M males are more efficient at mating with females, this fitness advantage could leave less females available for mating with Y^M males. Alternatively, we cannot rule out that females could be choosing to mate with III^M males over Y^M males. There were no effects of female strain (CS or IsoCS; $P = 0.99$) or nail enamel color applied to males ($P = 0.43$ and 0.18 for CS and IsoCS, respectively) on mating competition.

Temperature Effects on Pupal Emergence. The III^M males (CS) emerged at a higher frequency at all temperatures than Y^M males (IsoCS; Fig. 3). The 10% greater emergence of CS III^M males over IsoCS Y^M males at 28°C could explain an additional advantage of autosomal males in our cage study, because that was the temperature at which all cages and pupae were reared. The total percent emergence of CS flies was 92.4, 89.0, and 87.5% at 28, 16, and 4°C, respectively. The total percent emergence of IsoCS flies was 90.4, 83.6, and 84.4% at 28, 16, and 4°C, respectively.

Discussion

The three studies conducted all show III^M males have a greater fitness than Y^M males. These advantages include competing in cages with an initial frequency of 50% III^M and 50% Y^M males, mating competition for a female, and emergence of pupae held at 4, 16, or 28°C for 3 d. The III^M males seem to have greater fitness in every aspect tested over Y^M males. This would explain the stability of III^M males in the Florida population for the past 30 yr. The cage study showed that, even with migration of Y^M males from neighboring states (i.e., Y^M males in Alabama; Liu and Yue 2001), they would have lower fitness and possibly be driven out of the population as was seen in cage D by generation 5. This does not explain why there is a cline with Y^M males existing in northern populations (Franco et al. 1982, Tomita and Wada 1989, Hamm et al. 2005) or why populations with both Y^M and A^M males are stable (Hamm and Scott 2008, Kozielska et al. 2008). There are many other fitness characteristics to consider, but of those tested in this study, none showed an advantage for Y^M males. It was assumed that III^M males have a lower fitness in an environment with a cold stress because of the cline exhibited. In this study, more III^M males emerged after being held for 72–76 h at 4°C than Y^M males. However, 72–76 h is a relatively brief exposure to cold temperature and only one life stage was tested. Conditions in northern climates are much colder for longer periods of time. It could be that Y^M males have better survival in these cold environments over longer periods of time or that there are other factors responsible for the preservation of Y^M males in Maine.

In summary this is the first attempt using isogenic strains to determine fitness differences of Y^M versus III^M male house flies. The RAPD method developed to determine male sex determination was critical for these studies and could be used to distinguish between males of these two isogenic strains in future studies. The markers are not specific for all strains containing Y^M and III^M sex determination (unpublished data). There are many other characteristics that could be involved in greater fitness of one male type over the other. Longevity, development time, and overwintering effects could also play a large role in the selective advantage of one male over the other. It will be im-

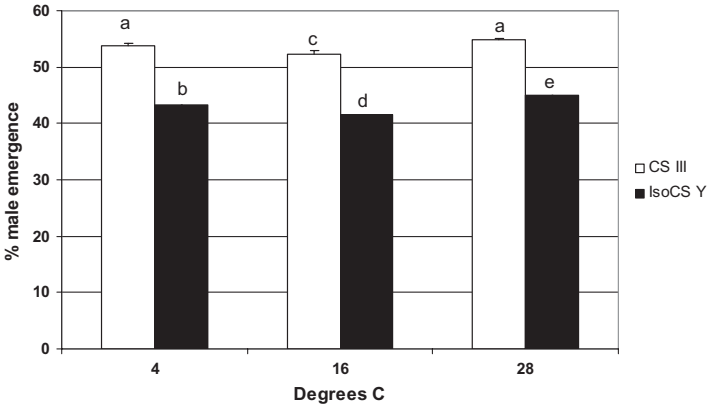

Fig. 3. Comparison of Y^M (IsoCSY) and III^M (CSIII) male house fly emergence from pupae held for 72–76 h at 4, 16, or 28°C. Different letters are statistically significant at $P < 0.01$.

portant to determine other selective advantages such as longevity, number of eggs produced, predator avoidance, or susceptibility to disease in an effort to explain the clines reported in various countries.

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