Ecological Indicators 96 (2019) 437–447

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

Reproductive performance of *Octopus maya* males conditioned by thermal stress

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ARTICLE INFO

Keywords:
Octopus maya
Sperm quality
Testis damage
Physiological condition
Multiple paternity
Reproduction

ABSTRACT

Observations of wild male *O. maya* suggest that temperatures below 27 °C favour their reproductive performance. From these observations we hypothesize that, as in females, the temperature modulates the reproductive performance of adult *O. maya* males. The study aimed to evaluate the physiological condition, reproductive success, and histological damage in testis of male *O. maya* exposed to thermal stress, to determine the implications of ocean warming over their reproductive performance. High temperatures (28–30 °C) negatively affect the growth and health of male *O. maya*. In octopuses maintained at 30 °C, as a consequence of the thermal stress we observed an increment in the haemocytes number, a reduction in the oxygen consumption rate, and an inflammatory process in the testis. The number of spermatozoa per spermatophore was not affected by temperature, but higher spermatophores production was observed at 30 °C. The paternity analysis showed that the offspring had multiple paternity with an average of 10 males contributing in a single spawn. The paternal contribution was affected by temperature with high, medium, or no paternal contribution in animals maintained at 24 °C (control group), 28 °C and 30 °C, respectively. The temperatures from 28 °C to 30 °C deeply affected the reproductive performance of *Octopus maya* males, suggesting that, as embryos, reproductive performance of adult males of this octopus species can be used as a tool for monitoring thermal changes in Yucatán Peninsula, located at the entrance of Gulf of Mexico.

1. Introduction

Aquatic environments are thermally heterogeneous in time and space. Organisms inhabiting these environments, specifically ectotherm organisms, show morphological, behavioural and physiological mechanisms (phenotypic plasticity) that give them adaptive capabilities to cope with environmental changes (Bozinovic and Pörtner, 2015; Deutsch et al., 2015; Piasečná et al., 2015; Pigliucci, 1996; Somero, 2010) Animal physiology, ecology, and evolution are affected by temperature and it is also expected that community structure will be

Abbreviations: ASP, Percentage of alive spermatozoa; B, Breeders; BW, Octopus total body weight; DF, Dilution factor; DGI, Digestive gland index; DGW, Digestive gland weight; DO, Dissolved oxygen; DS, Differentiation stratum; Ɛ, Extinction coefficient; F, Water flow rate; F_{IS}, Inbreeding coefficient; GSI, Gonadosomatic index; Hc, Hemocyanin concentration; H_o/H_e, Observed/Expected heterozygosity; hOp, Hemolymph osmotic pressure; $H_{W\text{-}E}$, Hardy–Weinberg equilibrium; L/D, Light/Dark; Na, Allele number; O, Offsprings; *O. maya*, *Octopus maya*; $O2_i/O_{2o}$, Oxygen concentration of the water inlet/outlet; OP, Osmotic pressure; OsmC, Osmoregulatory capacity; PS, Proliferative stratum; PVC, Polyvinyl carbonate; SCI, Spermatophoric complex index; SCW, Spermatophoric complex weight; SGC, Strata of germ cells; SGR, Specific growth rate; ST, Seminiferous tubules; STN, Spermatophores total number; TASC, Total number of alive spermatozoa; THC, Total haemocytes count; TSC, Total Number of spermatozoa; TW, Testis weight; VO_2, Oxygen consumption; WG, Weight gain; Wi/Wf, Initial/Final weight; wOp, Water osmotic pressure; ww, Wet weight; YP, Yucatan Peninsula

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https://doi.org/10.1016/j.ecolind.2018.09.036
Received 12 March 2018; Received in revised form 4 September 2018; Accepted 16 September 2018

strongly influenced by global warming (Nguyen et al., 2011). For example, temperature seemed to play the most important role in structuring the distribution of cephalopod body size along the continental shelves of the Atlantic Ocean (Rosa et al., 2012).

In the eastern region of the continental shelf of Yucatan Peninsula (YP), Mexico, a summer upwelling allows sub-superficial subtropical water from the Caribbean (between 150 and 200 m deep) to enter the shelf with temperatures between 16 °C and 22 °C (Enriquez et al., 2013). This cold water mass, besides functioning as an external temperature control for the shelf, transports nutrients which are used by primary producers (Enriquez et al., 2010). This upwelling affects only the eastern portion of the YP continental shelf provoking a summer thermal gradient that runs from the western to the eastern shelf from high to low temperatures, offering different environments to aquatic species of the zone (Zavala-Hidalgo et al., 2006, 2003; Ciencias de la atmósfera, http://uniatmos.atmosfera.unam.mx/ACDM/).

Octopus maya is endemic to the YP continental shelf and the most important octopus fishery in the American continent, with an annual production fluctuating between 8000 and 20,000 Tons (Galindo-Cortés et al., 2014; Gamboa-Álvarez et al., 2015; Markaida et al., 2016; SAGARPA, 2013). *O. maya* as an ectotherm organism is particularly temperature-sensitive (Noyola et al., 2013a,b) that can be affected in its morphology, behaviour, physiology and reproduction by changes in ambient temperature with spatio-temporal fluctuations (Avila-Poveda et al., 2015). Predictions of the thermal processes on the YP shelf indicate that sea temperatures may rise between 2.5 and 3 °C in the zone where upwelling has no effect (Enriquez et al., 2013; Saldívar-Lucio et al., 2015). Gamboa-Álvarez et al. (2015) observed that during the August-December fishing season, the greatest abundances of *O. maya* was found along the Campeche coast (western zone, without upwelling influence), where small octopus were fished; whereas, in the eastern zone, less abundances were recorded, but octopus with higher biomass were caught.

In laboratory conditions, at 31 °C the spawning of female *O. maya* was significantly reduced and only 13% of the total females (n = 32) spawned, while the few fertilized eggs (embryos) were not developed or died after two weeks (Juárez et al., 2015). It was observed that females exposed to a temperature decrease of 1 °C every 5 days and starting at 31 °C, only 87% spawned after temperatures reached less than 27 °C, and of these only 50% of the eggs laid (mean 530 eggs per spawn) were fertilized (Juárez et al., 2015). Those results suggested that temperature could be deleterious to sperm stored in the spermathecae of the oviductal glands, which play a crucial role in octopus reproduction (Olivares et al., 2017). At a later date, Juárez et al. (2016) found that juveniles performance from stressed females had lower growth rate and twice the metabolic rate than hatchlings coming from unstressed females, providing evidence that temperature stress experienced by females has consequences on the performance of hatchlings. Taking into consideration that *O. maya* wild population could be affected in summer when the benthic temperatures reach 30 °C, Angeles-Gonzalez et al. (2017) pustuled the hypothesis that that thermal condition causes migration of octopuses from western to eastern zone of the Yucatan Peninsula (YP) where upwelling events limit temperature increase. That migration was used to explain why reproduction occurs all the year in the eastern zone while in the western zone of YP only occurs in winter when temperatures are low (22–25 °C). When was analysed the multilocus microsatellite genotypes of wild *O. maya* across its distribution area to find out if the population is structured, and if the structure matches the mentioned thermal zones, was find that there is significant genic differentiation in the *O. maya* population that match with the two different thermal zones where *O. maya* is distributed. That results suggested that thermal differences between zones is the responsible of such genic structure differences (Juárez et al., 2018), suggesting that if these two subpopulations differ in features such as reproductive season, it is necessary to adjust management policies to the different population dynamics in each region to improve fishery productivity (Juárez et al., 2018).

To date, a small number of studies have investigated multiple paternity within cephalopods by using microsatellite markers demonstrating that multiple paternity could be a common characteristic in octopus species. Diverse studies have found at least two to four genetically distinct sires involved in the contribution to the progeny in *Graneledone boreopacifica*, *O. vulgaris* and *Euprymna tasmanica* (Voight and Feldheim, 2009; Quinteiro et al., 2011; Squires et al., 2014).

There is enough evidence demonstrating that temperatures higher than 27 °C have serious consequences on the reproductive performance and success of female *O. maya*. In this sense, new questions arise: As was observed in females, is 27 °C a thermal threshold for reproductive performance of *O. maya* males? Do *O. maya* males have the physiological mechanisms that allow them to compensate possible damages at temperatures higher than 27 °C? To address these questions, we designed a series of experiments to evaluate the effects of fixed temperatures (24 °C, 28 °C and 30 °C) on adult males of *O. maya* through assessment of their: i) Physiological condition, evaluating the specific growth rate, weight gain, digestive gland index, blood haemocytes and hemocyanin concentration, osmotic capacity and oxygen consumption; ii) Reproductive performance, evaluated through sperm quality and its relationship with histological characteristics of the testis, and iii) Reproductive success, estimated through the proportion of hatchlings generated by each male in each spawning. Wild adult females were mated with laboratory stressed males. Considering that multiple paternity can be present in *O. maya,* a paternity analysis implementing specific microsatellite markers was performed to assess the reproductive success of the experimental males.

To our knowledge, this is the first work that investigates the chronic thermal effect in the reproductive performance and success of male octopuses.

2. Material and methods

2.1. Ethics statement

In this study, octopuses were anesthetized with ethanol 3% in seawater at experimental temperatures (Estefanell et al., 2011; Gleadall, 2013) to induce narcotisation to enable humane killing (Andrews et al., 2013) in consideration of ethical protocols (Mather and Anderson, 2007), and the animaĺs welfare during manipulations (Moltschaniwskyj et al., 2007). Our protocols were approved by the experimental Animal Ethics Committee of the Faculty of Chemistry at Universidad Nacional Autónoma de México (Permit number: Oficio/FQ/CICUAL/099/15). We encouraged the effort to minimize animals stress and the killing of the minimum necessary number of animals for this study.

2.2. Animal capture and laboratory conditioning

Seventy-two wild *O. maya* adult males with body weight above 400 g were captured in the Sisal coast of the Yucatan Peninsula (21°9′55″N, 90°1′50″W), by using the local drift-fishing method known as “Gareteo” (Pascual et al., 2011; Solís-Ramírez, 1967). Male octopuses were caught during three collection trips from June to September of 2015. All males were anatomically mature with a well-developed reproductive system (Avila-Poveda et al., 2016). Octopuses were maintained in a 400-L black circular tank with seawater recirculation and exchange during the capture and then transported to the Experimental Cephalopod Production Unit at the Multidisciplinary Unit for Teaching and Research (UMDI-UNAM), Sisal, Yucatan, Mexico. Octopuses were acclimated for 10 d in 6 m diameter outdoor ponds provided with aerated natural seawater (26 ± 1 °C). The ponds were covered with black mesh reducing direct sunlight to 70%, and connected to seawater recirculation systems coupled to protein skimmers and 50 μmb bag filters. PVC 50 mm diameter open tubes were offered as refuges in proportion 2:1 per animal. Octopuses were fed individually

twice a day with a paste made with squid and crab meat at ratio of 8% of its body weight (Tercero et al., 2015).

2.3. Experimental design

After the conditioning period 69 adult male *O. maya* were randomly distributed in 80 L individual tanks at three different temperatures, 24 °C, 28 °C and 30 °C with n = 23 specimens per treatment, and mean weights of 584 ± 193 g ww, 692 ± 203 g ww, and 557 ± 160 g ww, respectively. Males were maintained in experimental conditions during 30 d and fed with the same paste used during the conditioning period. Seawater in tanks was maintained in a semi-closed recirculation system coupled with a rapid-rate sand filter and 36 ± 1 ppt salinity, dissolved oxygen higher than 5 mg L^{-1}, pH above 8, photoperiod of 12L/12D and a light intensity of 30 Lux cm^{-2}. For the experimental temperatures above 26 °C, seawater temperature was gradually increasing 2 °C per day until the experimental temperature was reached. Temperatures of 28 °C and 30 °C were controlled with 1800-Watt heaters connected to automatic temperature controllers, while temperature of 24 °C was controlled with a titanium chiller and the air conditioning of the experimental room.

2.4. Physiological condition

2.4.1. Specific growth rate and digestive gland index

We used 23 octopus adult males to evaluate physiological condition of animals exposed to experimental treatments. These animals were classified as PRE-mating, taking into account that they were only exposed to experimental temperatures for 30 d. Before measurements, animals were anesthetized with alcohol 3% in sea water at the actual experimental temperature; this procedure took 3–6 min. The organisms were considered anesthetized when the respiration was imperceptible (Gleadall, 2013). Afterwards, each octopus was weighted and a blood sample of 100–150 μL was drawn using a catheter inserted in the dorsal aorta. The sample was kept in ice until the haemocytes count. Once samples were obtained, octopus were euthanized cutting the brain in the middle of the eyes (Gleadall, 2013). Afterwards, the reproductive system and total digestive gland were extracted.

Total weight gain (WG) is the difference between the octopuses' wet weight at the beginning and the end of the experiment. Specific growth rate (SGR) was calculated as SGR = [(LnWf − LnWi)/t] ∗ 100, where Wf and Wi are the octopuses' final and initial wet weights, respectively, Ln is the natural logarithm and t is the number of experimental days. Survival was calculated as the difference between the number of animals at the beginning and at the end of the experiment. The Digestive gland index was calculated as: DGI = (DGW/Wf) * 100: where DGW = digestive gland weight in g (Cerezo Valverde et al., 2008).

2.4.2. Total haemocytes count and hemocyanin concentration (Hc)

Total haemocytes count (THC) was determined by processing the 10 μL of hemolymph sample immediately after extraction. The hemolymph sample was placed in TC10 counting slides with dual chambers and the readings were performed with a TC10™ automated cell counter (Bio-Rad). The hemocyanin concentration was measured by using 990 μL of TRIS 0.1 M (pH 8.0) and 10 μL of hemolymph. These procedures were triplicated. Hemocyanin measurements were performed using a spectrophotometer Genesys 10 with UV lamp (Thermo Scientific) in 1 ml UV cells at 335 nm of absorbance. The Hc concentration was calculated as: Hc = (mean Abs/Ɛ)/DF; where Abs = absorbance at 335 nm, Ɛ = extinction coefficient (17.26), and DF = dilution factor.

2.4.3. Osmoregulatory capacity (OsmC)

The osmotic pressure (OP) of 20 μL hemolymph samples were measured for every octopus in each treatment concurrently with the OP of three water samples in each treatment. OP was measured in a Micro osmometer 3MoPLUS (Advanced Instruments). The osmotic capacity was calculated as: OsmC = hOp − wOp; where hOp = hemolymph osmotic pressure and wOp = water osmotic pressure.

2.4.4. Oxygen consumption (VO_2)

The oxygen consumption (VO_2) was measured using a continuous flow respirometer where respirometric chambers were connected to a well-aerated, recirculating seawater system (Rosas et al., 2008). Eight male octopi per experimental condition were placed in 15 L chambers with an approximate flow rate of 5 L min^{-1}. All animals were allowed to acclimate to the chambers for 30 min before measurements were made. A chamber without an octopus was used to known the oxygen consumption of bacteria that could interfere in the final evaluation of the metabolic rate of the animals measured at each experimental temperature. Measurements of dissolved oxygen (DO) were recorded for each chamber (at entrance and exit) every minute during 4 h using oxygen sensors attached to flow cells, which were connected by an optical fibre to an Oxy 10 mini-amplifier (PreSens©, Germany). The sensors were calibrated for each experimental temperature using saturated seawater (100% DO) and a 5% sodium sulphate solution (0% DO).

The oxygen consumption (VO_2) was calculated as $VO_2 = [(O_{2i} - O_{2o}) * F]/Bw$; where O_{2i} = oxygen concentration of the water inlet (mg/L^{-1}), O_{2o} = oxygen concentration of the water outlet in each experimental chamber (mg/L^{-1}), F = water flow rate (L/h^{-1}), BW = octopus total body weight (g).

2.5. Reproductive performance

2.5.1. Reproductive indexes and sperm quality

To establish the sexual maturity and reproductive activity of the experimental octopuses during 30 d of thermal exposure, the following indexes were estimated:

The Gonadosomatic index, GSI = (TW/BW) * 100; Spermatophoric complex index: SCI = (SCW/BW) * 100; Maturity coefficient: MC = [(TW + SCW)/BW] * 100; where TW = testis weight (g); SCW = spermatophoric complex weight (g); BW = total body weight (g) (Krstulovic-Sifner and Vrgoc, 2009; Rodrigues et al., 2011; Sivashanthini et al., 2010).

The total number of spermatophores (STN) for each Needhaḿs sac was counted. Three spermatophores per octopus were taken to evaluate the total number of spermatozoa (TSC), as well as the number and percentage of alive (TASC and ASP) and dead spermatozoa for each experimental treatment. Spermatophores were homogenized in 2 ml of Ca^{2+} free solution. Then 10 μL of the homogenate was mixed with 4% tripan blue (v/v). Readings were performed in a TC_{10} Automated Cell Counter (Bio-Rad) with 10 μL of the mix.

2.5.2. Testis histology

A portion of the gonad of approximately 1 cm^3 was taken by performing a perpendicular cut to the tunica albuginea (the fibrous connective membrane that covers the testis, "testis wall"). That portion of the gonad was fixed in Davidson's fixative for 3 d (Elston, 1990), rinsed in 70% ethanol, dehydrated in an ethanol series, cleared in Ultraclear®, permeated and embedded in Paraplast® tissue embedding medium (m.p. 56 °C). Sections of 5 μm were stained with Harris's Hematoxylin and Eosin regressive method (Howard et al., 2004). Slide examinations were performed at 400× and digital images were obtained with a digital imaging system (Micrometrics® SE Premium 4.4 software, ACCU_SCOPE) mounted on an Olympus H30 compound microscope.

Twenty seminiferous tubules (ST) close to the tunica albuginea found in longitudinal sections were randomly selected and two widths and three heights were measured: width of ST and lumen, height of the strata of germ cells (SGC: spermatogonia, spermatocytes of 1st and 2nd order, round spermatid, ovoid spermatid, and elongated spermatid), height of proliferative stratum (PS: spermatogonia, spermatocytes of 1st and 2nd order, and round spermatid), and height of differentiation

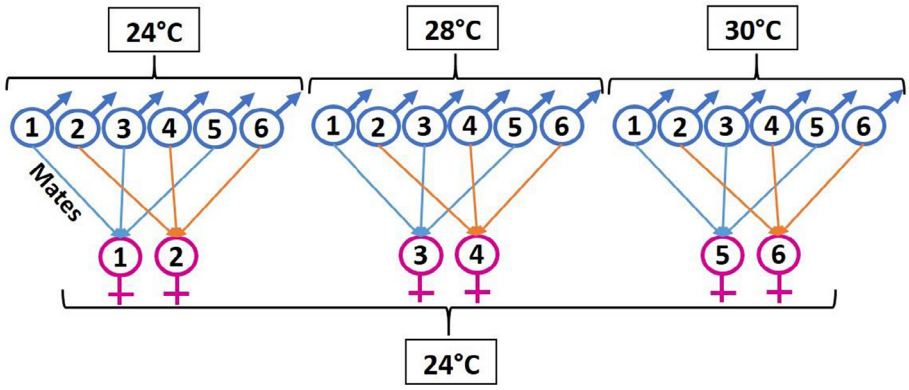

Fig. 1. Mating system used in *Octopus maya* per experimental temperature. Males maintained at different experimental temperatures (24 °C, 28 °C and 30 °C) during 30 d were mated with females at 24 °C. The matings were done one by one for each temperature. Copulation lasted 4 to 6 h and between each mating the females had a recovery time of 4 d until the next mating. The females were acclimated for 15 d at 24 °C until mating.

stratum (DS: ovoid spermatid and elongated spermatid), where SGC = PS + DS (Fig. S1). The total relative surface area measured was then considered to the nearest 5 mm^2. The percentage of disorders in the area of germinal cells such as completely acidophilic bodies, or with basophilic material, and vacuolated basal compartments were calculated.

2.6. Male reproductive success

2.6.1. Mating protocol

Six of the 23 octopuses for each experimental temperature were mated with two females in such form that a sexual proportion of 3:1 males-females was ensured in each experimental temperature. All the *Octopus maya* females were maintained in 80 L natural seawater tanks, but at a 24 °C constant temperature. This experiment was done trying to ensure that each female was mated with at least three different males from the each experimental temperature (Fig. 1). Male octopuses from each experimental condition, were acclimated during 30 min in individual tanks until reached 24 °C and then placed in the female tanks. Males were allowed to mate during 4 to 6 h and then returned to their experimental tank. Mating finish was established when the male separated the hectocotylus out of the female cavity and stay away from her. Males used in the mating protocol were sacrificed 12 h after mating following the protocol previously described. Those males were considered POST-mated and classified as POST.

Pregnant females were maintained in individual tanks until spawning, and fed twice a day. After spawning, wet weight was recorded. Each spawning was placed in an artificial incubator (Rosas et al., 2014) during 45–50 d, with a range temperature of 22 °C to 24 °C, and constant salinity, pH, aeration, and seawater recirculation. Data of the number of eggs per spawn, number of hatchlings, hatchlings wet weight, deformities, fecundity, and survival of hatchlings after 10 d fasting were recorded. To evaluate the quality of hatchlings obtained from females mated with males exposed at different experimental temperatures, hatchlings survival was evaluated by placing 20 juveniles in PVC tubes individualized without feeding during 10 d (Rosas et al., 2014).

2.7. Statistical analyses

Data were expressed as mean ± SD. Differences among values of each measurement (widths and heights) throughout the treatments (temperature and condition PRE-POST) were evaluated by two-way ANOVA followed by Fisher LSD (least significant difference) tests. Data transformation were applied to obtain normality and homocedasticity to fulfill the ANOVA assumptions (McCune et al., 2002; Zar, 2010). Statistical analyses were carried out using STATISTICA7® (StatSoft). Statistical significance was accepted if $P < 0.05$.

No significant differences were found between the PRE and POST reproductive conditions among all tested parameters; therefore, the data of the 23 tested octopuses were used to calculate the mean for the different parameters and only thermal exposure was considered as the main effect factor.

2.8. Paternity analyses

2.8.1. DNA extraction

The DNA of 47 hatchlings per spawn, for a total of 282, and breeders, six females and 17 males, was extracted from arm tissues. Approximately 30 mg of tissue were homogenized with mortar and pestle, adding liquid nitrogen. DNA was extracted using the DNeasy® Blood and Tissue kit (Qiagen) following the supplier instructions. The concentration and purity of each DNA sample were measured with a Nanodrop (Thermo-Scientific) spectrophotometer. The DNA integrity was assessed with an electrophoresis in agarose gel (1%) at 85 V for 40 min.

2.8.2. Microsatellite amplification

To obtain the hatchlings and breeders genotype, five polymorphic microsatellite loci previously characterized (Juárez et al., 2013; Table 1) were selected for polymerase chain reaction (PCR) amplification. PCR primers were marked with 6FAM, VIC, PET, and NED fluorescent dyes (Applied Biosystems) for subsequent fragment analysis. The PCR for each microsatellite was performed in a thermal cycler CFX96 Touch™ (Bio-Rad), on 96-well plastic wells. The 15 µL reaction volumes contained: 3 µL Buffer (5X), 0.9–1.5 µL $MgCl_2$ (25 mM), 0.3 µL dNTP (10 mM), 0.15 µL of each primer (10 µM), 1 µL DNA (40 ng/µL), 8.3–9.425 µL H_2O depending on each locus (specific PCR conditions of each locus in Table 1), and 0.075 µL of Go Taq Flexi DNA polymerase (5 u/µL, Promega). The general amplification program was: 2 min at 94 °C; followed by 35 cycles of 30 sec at 93 °C, specific alignment time at specific Tm (Table 1), and 30 sec at 72 °C; finally an elongation step was added (10 min at 72 °C). Positive and negative controls were included in each plate. The PCR amplicons were verified by electrophoreses in agarose gels (1.5%) at 85 V for 40 min. The amplicons marked with different fluorophores obtained from the same sample,

Table 1
Primer sequences, characteristics and PCR conditions for amplification of 5 microsatellite loci of *O. maya* (Juárez et al., 2013).

Locus	Multiplex	Repeat motif	Ta (°C)	T	$MgCl_2$ (mM)	Primer tag
Omy2-0	I	$(GT)_{17}$	58.5	30″	2	6FAM
Omy2-07	I	$(GAT)_{18}$	57	30″	1.5	NED
Omy4-01	I	$(TATG)_9$, $(TATC)_8$	61.5	30″	2	PET
Omy4-11	II	$(GT)_{10}$, $(GA)_6$	60	50″	2.5	6FAM
Omy4-18	II	$(ATGT)_9$	56.8	30″	1.5	VIC

I – II Number of multiplex; Ta – optimized annealing temperature; T – annealing time.

were multiplexed for fragment analysis in an AB genetic analyzer (Applied Biosystems).

2.8.3. Fragment analyses and genotyping

Fragment analyses were performed in the AB 3730xl genetic analyzer (Applied Biosystems) at the Illinois University Roy J. Carver Biotechnology Center (USA). The allele size in each sample was assigned using the PEAK SCANNER software (Applied Biosystems). The multilocus genotype of each sample (offsprings and breeders) was registered to build a data matrix.

2.8.4. Parentage and data analyses

The paternity analyses were conducted using two different softwares COLONY 2.0.6.3, and GERUD 2.0. COLONY estimates the maximum number of sires in the spawn using a maximum-likelihood method to assign parentage and sibship groups, if the potential fathers were not sampled the program reconstructs the genotypes (Jones and Wang, 2010). For each spawn, the potential father's genotypes were inferred, providing the mother, the candidate fathers, and offsprings genotypes as input data for the analysis. If the genotypes of the candidate males did not appear in the inferred father genotypes (paternity), it was assumed that the father was a wild male octopus. GERUD determines the minimum number of paternal genotypes that are necessary to produce the genotypes of the progeny in the spawn based on the Mendelian segregation laws, and the allele frequencies in the spawns, considering consistent maternal genotypes (Jones, 2005). For each spawn, the maternal and offsprings genotypes were used as input for the analysis. Five microsatellite loci were used in the analysis; in some cases loci with missing data were discarded. A correlation between the number of inferred fathers and the experimental conditions was performed.

Observed and expected heterozygosity (H_o and H_e, respectively) of breeders and offsprings, Hardy–Weinberg equilibrium ($H_{W\text{-}E}$), and inbreeding coefficient (F_{IS}) were obtained using ARLEQUIN 3.5.2.2 software (Excoffier et al., 2005). The FIS index was estimated using the analysis of molecular variance (AMOVA) with 1000 permutations. The number of alleles and allele frequencies (Table S1) were obtained with the ARLEQUIN software.

3. Results

3.1. Physiological condition

Total weight gain (WG) and SGR (% d^{-1}) were affected by temperature (Table 2; $P < 0.05$). Total WG of animals maintained at 24 °C and 28 °C were 9 times higher than the observed in octopuses maintained at 30 °C. In consequence a SGR 6 times higher was obtained in animals maintained at 24 °C and 28 °C than those maintained at 30 °C (Table 2). We observed that octopuses exposed to 30 °C not only lost weight but also reduced their food ingest intermittently during the 30 d exposure period. The temperature also affected the DGI (Table 2). The DGI of animals maintained at 24 °C was 58% higher than those obtained in octopuses exposed at 28 °C and 30 °C (Table 2; $P < 0.05$).

Blood parameters were also affected by temperature. A higher concentration of THC was recorded in octopuses exposed to 30 °C ($2.5 \times 10^6 \pm 1.5 \times 10^6$ cells/ml) in comparison to organisms maintained at 24 °C and 28 °C (Table 2; $P < 0.05$). The Hc was significantly lower ($P < 0.05$) at 28 °C (1.84 mmol/L) than that observed in animals maintained at 24 °C and 30 °C (2.10 and 2.27 mmol/L; Table 2; $P < 0.05$). Considering that there were no statistical differences between OsmC values obtained in experimental animals, a mean value of 415 ± 85 mOsm kg^{-1} was calculated (Table 2; $P > 0.05$). Temperature affected the routine metabolism of male *O. maya* with values 42% lower in animals maintained at 30 °C (0.02 mg O_2 h^{-1} g^{-1}) than those observed in animals maintained at 24 °C or 28 °C (0.03 mg O_2 h^{-1} g^{-1}; Table 2; $P < 0.05$).

3.2. Reproductive performance

Temperature did not affect the spermatozoa content per spermatophore (TSC, TASC, and ASP, Table 3; $P > 0.05$). In contrast an increment of the STN-PRE with temperature was detected with lower values in animals maintained at 24 °C (84 spermatophores $animal^{-1}$) than those observed in octopuses exposed to 28 °C or 30 °C (mean value 129 spermatophores $animal^{-1}$; $P < 0.05$). The STN-POST also was affected by temperature with low values in animals maintained at 24 °C and 28 °C (mean value 52 spermatophores $animal^{-1}$) than those observed in octopuses maintained at 30 °C (108 spermatophores $animal^{-1}$; Table 3; $P < 0.05$).

The testis and the spermatophoric complex mean weights (TW and SCW) were not affected by temperature; mean values of 7.3 and 7.4 g $animal^{-1}$ can be calculated for male *O. maya* sampled in this study ($P > 0.05$; Table 3). The GSI, SCI, and MC were affected by experimental temperature with significantly higher values in animals maintained at 30 °C than observed in octopuses exposed at 24 °C and 28 °C (Table 3; $P < 0.05$).

With increasing temperature, a dilation of the seminiferous tubules and their lumen were evident, from 24 °C to 28 °C increasing 50–60 μm, while from 28 °C to 30 °C the dilation increased another 80–100 μm. Despite the expansion of the seminiferous tubules and lumen, each one

Table 2
Physiological condition of *O. maya* males exposed to chronic thermal stress.

	Temperature			Statistics
	24 °C	28 °C	30 °C	ANOVA
Wi	584 ± 193 ^{ab}	692 ± 203 ^{a}	557 ± 160 ^{b}	*
Wf	836 ± 216 ^{a}	944 ± 202 ^{a}	587 ± 179 ^{b}	*
WG	252 ± 113 ^{a}	265 ± 132 ^{a}	29 ± 173 ^{b}	*
SGR	1.14 ± 0.44 ^{a}	1.07 ± 0.57 ^{a}	0.18 ± 1.00 ^{b}	*
DGI	4.28 ± 0.65 ^{a}	3.09 ± 0.70 ^{b}	2.34 ± 1.11^{c}	*
DGW	35.4 ± 8.6 ^{a}	28.8 ± 7.9 ^{b}	14.3 ± 8.8^{c}	*
THC	$1.5 \times 10^6 \pm 7.9 \times 10^5$ ^{a}	$2.2 \times 10^6 \pm 8.2 \times 10^5$ ^{a}	$2.5 \times 10^6 \pm 1.5 \times 10^6$ ^{b}	*
Hc	2.10 ± 0.34 ^{a}	1.84 ± 0.27 ^{b}	2.27 ± 0.64 ^{a}	*
OsmC	416 ± 79 ^{a}	428 ± 66 ^{a}	402 ± 108 ^{a}	n.s.
VO_2	0.036 ± 0.016 ^{a}	0.033 ± 0.004 ^{a}	0.020 ± 0.003 ^{b}	*
T	30	30	30	–

Data as mean of 23 octopus ± SD per temperature, except for OsmC data that was analyzed with the mean of eight individuals per temperature. Values on the same line and different superscripts are significantly different (n.s. = not significant ($P > 0.05$); *$P < 0.05$). Wi, Initial weight (g); Wf, Final weight (g); WG, Weight gain (g); SGR, Specific Growth Rate (%); DGI, Digestive Gland Index (%); DGW, Digestive gland weight; THC, Total hemocytes count (Cells/mL); Hc, Total hemocyanin (mmol/L); OsmC, Osmoregulatory capacity (mOsmKg^{-1}); VO_2, Oxygen consumption (mgO_2 h^{-1} g^{-1} ww); T, time of exposure (d).

Table 3
Reproductive performance and sperm quality indicators calculated for *O. maya* males exposed to chronic thermal stress.

	Temperature			Statistics
	24 °C	28 °C	30 °C	ANOVA
N	23	23	23	–
TSC	$1.3 \times 10^6 \pm 5.7 \times 10^5$ [a]	$1.4 \times 10^6 \pm 4.7 \times 10^5$ [a]	$1.4 \times 10^6 \pm 3.5 \times 10^5$ [a]	n.s.
TASC	$5.2 \times 10^5 \pm 2.3 \times 10^5$ [a]	$5.5 \times 10^5 \pm 1.9 \times 10^5$ [a]	$5.3 \times 10^5 \pm 1.3 \times 10^5$ [a]	n.s.
ASP	37.4 ± 3.4 [a]	36.7 ± 4.9 [a]	37.1 ± 3.8 [a]	n.s.
STN[1]	84 ± 20 [a]	115 ± 30 [b]	142 ± 63 [b]	*
STN[2]	54 ± 32 [a]	49 ± 17 [a]	108 ± 40 [b]	*
TW	7.66 ± 1.93 [a]	7.81 ± 1.97 [a]	6.34 ± 2.72 [a]	n.s.
SCW	6.70 ± 3.09 [a]	7.63 ± 1.99 [a]	7.87 ± 2.10 [a]	n.s.
GSI	0.93 ± 0.15 [a]	0.84 ± 0.19 [a]	1.11 ± 0.51 [b]	*
SCI	0.78 ± 0.22 [a]	0.83 ± 0.24 [a]	1.46 ± 0.60 [b]	*
MC	1.70 ± 0.32 [a]	1.66 ± 0.32 [a]	2.57 ± 0.91 [b]	*

Values on the same line and different superscripts are significantly different (– not applicable; n.s. = not significant ($P > 0.05$); * $P < 0.05$). N = number of tested octopus; TSC, Total sperm count (cells ml^{-1} spermatophore^{-1}); TASC, Total alive sperm count (cells ml^{-1} spermatophore^{-1}); ASP = Alive sperm percentage (%); STN, Spermatophores total number ([1]-PRE, [2]-POST); TW, Testis weight (g); SCW, Spermatophoric complex weight (g); GSI, Gonadosomatic index (%); SCI, Spermatophoric complex index (%); MC, Maturity coefficient (%).

of the two strata (proliferative and differentiation) forming the area of germ cells showed no significant change in height with increasing temperature ($P > 0.05$), except at 30 °C where shrinkage of about 20 µm was observed, mostly the proliferative stratum (spermatogonia, spermatocytes of 1st and 2nd order, and round spermatid). All treatments showed completely acidophilic bodies in all strata of germ cells in an order of 3%–5%, except octopuses treated at 30 °C, which showed a 4-fold of these completely acidophilic bodies compared to the other treatments (Fig. 2). At 30 °C we observed acidophilic bodies with basophilic material, and vacuolated basal compartments (Fig. 3).

3.3. Male reproductive success

3.3.1. Fertilization

Fertilization rate was apparently not affected by temperature. All the females mated with males from experimental temperatures spawned normal eggs that developed as embryos and hatched without deformities. Egg fertilization fluctuated between 53% and 92% with no apparent relationship with the experimental temperature experienced by males (Table 4). Also, hatchlings survival after the 10 d fasting was high with percentages that oscillated between 85% and 100%. Kruskal-Wallis ANOVA did not show significant differences in the reproductive capacity of the females mated with the males exposed to different temperatures.

3.4. Paternity analyses

All the microsatellite loci used in this study were polymorphic and correctly amplified in all samples, showing a high level of genetic diversity (Table 5). Fifty one alleles were detected from 267 individuals (B- Breeder, O- Offspring). N_a ranged from four to 9 in B and 6 to 12 in O per locus. H_o ranged from 0.35 to 0.83 in B and 0.42 to 0.77 in O, respectively; and H_e ranged from 0.43 to 0.85 in B and 0.42 to 0.81 in O, respectively. F_{IS} ranged from −0.187 to 0.202 and 0.009 to 0.075 in B and O, respectively. F_{IS} averages were 0.007 with a P-value of 0.484 in B and 0.037 with a P-value of 0.014 in O, as a whole. $H_{W\text{-}E}$ performed among 10 locus for breeders-offsprings combinations, revealed a significant deviation at four loci ($P < 0.05$). These four loci were Omy2-0, Omy2-07, Omy4-01, and Omy4-11 in O, while B were within $H_{W\text{-}E}$. In the case of Omy4-18 were within $H_{W\text{-}E}$ in B and O (Table 5).

After analyzing the mother's genotype in each spawn, it was observed that some offspring did not correspond to the mother. This happened because octopus hatchlings are able to *escape* from their original incubator and *jump* into another one. These hatchlings, together with the samples with undetectable signals in the fragment analysis, were excluded from the parentage analysis.

Fathers were assigned to 244 octopus juveniles for which the mother was known. The results obtained with GERUD and COLONY revealed evidence of high levels of multiple paternity in all analyzed spawns.

The estimated minimum number of sires from the GERUD analyses ranged from three to five, with an average of 4 sires per spawn (Table 6, Fig. 4A). The mean maximum number of sires estimated with COLONY was 10 per spawn.

According to the parentage analysis using GERUD, when the males were exposed at 24 °C, only one experimental male contributed to the progeny (S24-1 and S24-2 in both the 1st male; Table 6, Fig. 4A); these males were the sires of nine and 16 offsprings, with a contribution of 19.6% and 38.1%, respectively, of the analyzed progeny. When the males were exposed at 28 °C, one experimental male was identified as potential sire of 9 offspring (S28-1 the 2nd male), contributing with 20.5% of the analyzed progeny (Table 6, Fig. 4A). In the case of the spawn S28-2 paternity could not be assigned. When males were acclimated to 30 °C, they had no contribution to the progeny (S30-1 and S30-2), but the minimum number of sires were 5 and 3, respectively. It was assumed that under this experimental condition all progeny belongs to wild males (Table 6, Fig. 4A).

The COLONY analysis results showed that when the males were exposed at 24 °C for 30 d, one to two experimental males contributed to the progeny with 19.6% and 21.4%, respectively, of the analyzed progeny (S24-1 the 1st one with 9 offspring, and S24-2 the 2nd and 3rd male with 5 and 4 offspring, respectively; Table 6, Fig. 4B). When the males were exposed at 28 °C, one experimental male was identified with 6 offspring and a parental contribution of 13.6% (S28-1). In the S28-2 spawn no sires were identified (Table 6, Fig. 4B). Males exposed at 30 °C, showed no contribution to the progeny. It was assumed that all the offspring correspond to wild male octopuses.

The COLONY analysis results also showed that independently of the maximum number of sires that explains the progeny, there are at least four males which contributed with the 57.1% of the total progeny per spawn, and the other 42.9% is distributed among the remaining parents (Fig. 4B).

4. Discussion

Previous studies showed that temperature modulates the reproductive capacity of *O. maya* wild populations, reducing the functional maturity and SCI (%) when environmental temperature in the YP continental shelf is around 30 °C (Angeles-Gonzalez et al., 2017). The present study was designed to evaluate if temperatures higher than

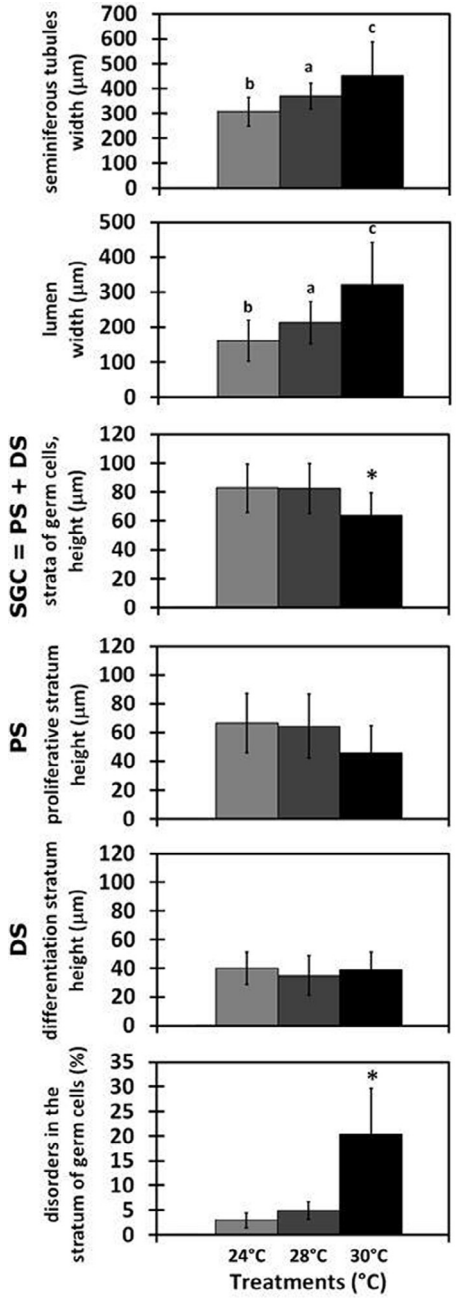

Fig. 2. Morphological changes in the germ cells strata (SGC = PS + DS), and the seminiferous tubules lumen during experimental thermal stress. Values are mean ± SD. Different letters indicate significant differences among treatments and asterisks denote significant differences from all other treatments at $P < 0.05$.

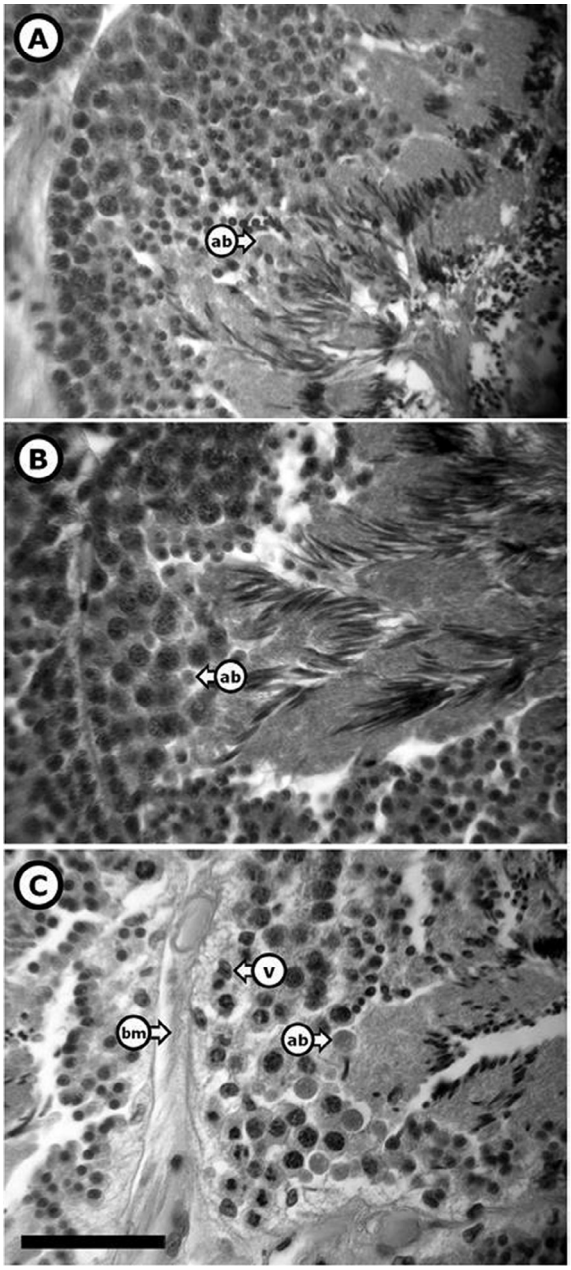

Fig. 3. Cross sections photomicrographs of *Octopus maya* seminiferous tubules during the chronic thermal stress. Treatments are: A) 24 °C, B) 28 °C and C) 30 °C. ab- acidophilic bodies, bm-basement membrane of the seminiferous tubule, v- vacuole in the basal area. General structure followed scheme from Fig. 2. Scale bars are 50 µm.

27 °C affect the reproductive capacity and success of male *O. maya* as observed when females and their embryos were exposed to thermal stress (Juárez et al., 2016, 2015; Sanchez-García et al., 2017). Results obtained in the present study, demonstrate that temperature of 30 °C affected negatively growth rate. For the digestive gland index of the adult *O. maya* males a negative effect was observed at temperatures from 28 °C to 30 °C. *O. maya* males exposed to 30 °C showed intermittent feeding, possibly as a consequence of the exposure to high temperatures, as reported in *O. pallidus* (André et al., 2008). The deleterious effect of temperature on the digestive gland could directly affect the reproductive performance because most of the energy that is directed to reproduction comes from this organ. At the same time, an increment of haemocytes, and a reduction on VO_2 were registered, indicating that several physiological mechanisms were affected in this thermal condition. In mollusks, in the absence of a specific immune system, the immune response is mediated by circulating haemocytes and molecular effectors that allow a rapid and effective response to stressors. In bivalve mollusks such as *Chamelea gallina* exposed to 30 °C, and cephalopods such as *Eledone cirrhosa* it was observed an increment in the circulating haemocytes (THC) when the organisms were exposed to different stressors, as observed in *O. maya* males (Malham et al., 2002; Monari et al., 2007).

Octopuses are aquatic ectotherms, an increment in temperature provokes an increment in the energetic demands that are essentially covered in first instance to maintain the homeostasis, even if the cost reduces growth (Sokolova et al., 2012). In adult *O. maya* males a reduction of the oxygen consumption and growth jointly with a decrease

Table 4
Reproductive capacity of *O. maya* males exposed at different experimental temperatures for 30 d.

	Males temperature (°C)		
	24 °C	28 °C	30 °C
Male Wet weight, g	699 ± 53	780 ± 160	519 ± 143
Reproductive success after mate:			
Female 1			
Spawn ID	S24-1	S28-1	S30-1
Wet weight, g	597	790	787
Mating time, h	4	5	6
Eggs per spawn	737	797	446
Number of hatchlings	537	509	411
Hatchlings wet weight, g	0.11 ± 0.02	0.12 ± 0.01	0.11 ± 0.01
Survival after 10 d fasting, %b	90	95	100
Deformities	0	1	2
Fecundity, %	73	64	92
Female 2			
Spawn ID	S24-2	S28-2	S30-2
Wet weight, g	628	1047	553
Mating time, h	4	5	6
Eggs per spawn	772	782	481
Number of hatchlings	518	418	424
Hatchlings wet weight, g	0.13 ± 0.01	0.11 ± 0.01	0.09 ± 0.01
Survival after 10 d fasting, %b	90	90	85
Deformities	8	1	0
Fecundity, %	67	53	88

Females maintained at temperature 24 °C.
Mating time as mean mating time of six males per temperature.
Mated males: 6 per treatment; Mated females: 2 per treatment; Mated males per female (N = 3); Number of hatchlings weighed: 50 per female; Number of hatchlings to evaluate survival after 10 d fasting period: 20 per female.

Table 5
Summary statistics of five microsatellite markers in *O. maya* males.

Locus	N_a	H_o	H_e	$P_{H\text{-}W}$	F_{IS}
Omy2-0					
B	9	0.74	0.85	0.367	0.128
O	12	0.77	0.81	0.000**	0.053
Omy2-07					
B	4	0.35	0.43	0.258	0.202
O	12	0.51	0.52	0.000**	0.009
Omy4-01					
B	9	0.83	0.74	0.542	−0.115
O	12	0.69	0.71	0.000**	0.018
Omy4-11					
B	6	0.78	0.66	0.792	−0.187
O	6	0.60	0.65	0.004*	0.075
Omy4-18					
B	4	0.48	0.51	0.669	0.064
O	7	0.42	0.42	0.083	0.011
Mean					
B	6.4	0.64	0.64	–	0.018
O	9.8	0.60	0.62	–	0.033

P-value calculated by using a Markov chain, performed in Arlequin 3.5.2.2.
B - Breeders (n = 23); O - Offsprings (n = 244); N_a, Allele number; H_o, Observed Heterozygosity ; H_e, Expected Heterozygosity; $P_{H\text{-}W}$, P-values for Hardy-Weinberg equilibrium; F_{IS}, Inbreeding coefficient. *P < 0.01; **P < 0.001.

on DGI (%) was observed in animals maintained at 30 °C. In *Sepia officinalis* it was observed that the oxygen consumption of animals from the English channel acclimated to 21 °C showed a metabolic rate lower than observed in cuttlefish acclimated to 15 °C (Oellermann et al., 2012). That pattern of thermal acclimation was explained by taking into account that a suppression of oxygen consumption rates in organs other than the hearts (e.g. digestive gland, mantle, or even reproductive tissues) could be occurring in this species. Although the tissue oxygen consumption was not measured in this study, we can hypothesize that as in cuttlefish, in *O. maya* there are compensatory mechanisms that reduce food ingestion and digestive gland metabolism to save energy, allowing the key organs such as the heart, to maintain the homeostasis of the animal, at least temporarily (Oellermann et al., 2012; Marshall and McQuaid, 2010; Fusi et al., 2016).

From a reproductive point of view, the 30 °C temperature treatment affected various levels of the testis organization: dilation of seminiferous tubes, shrinkage of the proliferative stratum where spermatozoa are synthetized, high quantity of acidophilic bodies, and a general disorder in the organization of the germinal tissue. Previous studies show that temperatures higher than 27 °C affected the reproductive capacity and success of *O. maya* females (Juárez et al., 2016, 2015; Sanchez-García et al., 2017). However, this is the first time that the effect of temperature on the reproductive capacity and success of octopus males is documented through histology of the testis and paternity inference. Our observations show that a temperature of 30 °C restricts the reproductive capacity and success of *O. maya* males via the possible production of a great number of spermatids with some kind of damage, identified from acidophilic bodies observed in testis. There are at least three hypothesis that could be postulated to explain why increase in spermatophores and spermatozoids in animals exposed at 30 °C did not contributed to impregnate females:

1) When impregned, spermatozoids cannot reach the oviductal gland due to movement limitations derived of some type of damage on the energy pathway in the cell;
2) There is a kind of damage in the spermatozoids that provoked some kind of damage at DNA/RNA level that affect its capacity to fecundate the oocytes (Histone and/or protamine damage (Gimenez-Bonafé et al., 1999) and
3) *O. maya* female can recognize, through some chemical signals, the best spermatozoids allocated in the oviductal gland using only those to fecundate the oocytes.

Temperature of 30 °C affected the structures of reproductive tissues in the adult males, provoking an inflammatory process in the testis and a higher disorder at the tissues than that observed in animals maintained at 24 °C. An intermediate condition was observed in animals maintained at 28 °C, suggesting that this may be a thermal threshold for reproduction of male *O. maya*. While temperature did not affect the number of spermatozoa per spermatophore, a higher production of spermatophores was observed in animals maintained at 30 °C. This suggests that despite the structural damage caused by temperature, animals responded by allocating enough energy to increase their reproductive potential. This could be a reproductive strategy to ensure the preservation of the species, through the formation of a greater number of spermatophores. Although we don't know if there is a direct relationship between quantity of live sperms and fertilization rate in *O. maya*, it is possible to think that a higher GSI could be activated as a compensatory mechanism to reduce the effects of changes in the testis structure due to thermal stress, increasing the fecundity probability of thermal stressed animals (Parker, 2016).

The analysis of six spawns with five different microsatellite loci in the progeny of six females confirmed the presence of multiple paternity in *O. maya*. A minimum number of four and a maximum of 10 males were estimated to contribute to the progeny. This conserved reproductive strategy has been observed in other octopod species such as *Graneledone boreopacifica* (Voight and Feldheim, 2009), *Enteroctopus dofleini* (Larson et al., 2015), *O. vulgaris* (Quinteiro et al., 2011), *O. oliveri* (Ylitalo-ward, 2014) and *Euprymna tasmanica* (Squires et al., 2014). It was also observed that the last mated experimental male had no parental contribution in any spawn, with exception of male S24-2, whose parental contribution was lower than that of the other males

Table 6
Number of sires assigned with the paternal analysis for each spawn of *O. maya* using COLONY and GERUD.

Spawn	MET (°C)	Loci	AO	IF	EF	WF	EO	WO	PC (%)
S24-1	24	5/5	46	4/7	1/1	3/6	9/9	37/37	19.6/19.6
S24-2	24	5/5	42	5/9	1/2	4/7	16/9	26/33	38.1/21.4
S28-1	28	5/5	44	5/11	1/1	4/10	9/6	35/38	20.5/13.6
S28-2	28	5/5	34	NA/12	NA/0	NA/12	NA/0	NA/34	NA/0
S30-1	30	5/5	42	5/10	0/0	5/10	0/0	42/42	0/0
S30-2	30	5/5	36	3/12	0/0	3/12	0/0	36/36	0/0
Mean				4/10					

Data obtained from GERUD/COLONY respectively.
MET – Males exposure temperature; AO - Analyzed Offsprings; IF – Inferred fathers; EF – Experimental fathers; WF – Wild fathers; EO – Experimental offsprings; WO – Wild offsprings; PC – Paternal contributions.
NA – Not applicable, exceeded the sire number assessable (six) using GERUD.

involved. Contrary to the pattern of the last sperm male precedence observed in *Octopus bimaculoides* and *Octopus minor* (Mohanty et al., 2014; Bo et al., 2016), in *O. maya*, the last male to copulate is not the best genetically represented in the offspring. The pattern identified in *O. maya* coincides with the pattern of first male precedence observed in *O. oliveri* (Ylitalo-ward, 2014). Indeed, under optimal conditions (24 °C) the experimental males contributed with an average of 57.1% of the total parental contribution for each spawning, regardless of the order of mating. However, several studies have shown that spermatic precedence is influenced by the order of mating, due to sperm competition, or mediated by female cryptic choice (Hirohashi and Iwata, 2016; Iwata et al., 2005; Quinteiro et al., 2011).

Temperature increase plays an important role in the parental contribution (reproductive success) of *O. maya* due to the fact that in the spawning of stressed parents (28 °C) a reduction in the parental contribution was observed. This was more evident at 30 °C where no contribution of the experimental males was found, independent of the mating order.

Temperature affected the growth and the metabolism of *O. maya* males by reducing the food ingested and the digestive gland index; as a consequence, the organism directed available energy to reproduction. Males under stress conditions produced a greater number of spermatophores. Nevertheless, this strategy seems to be insufficient given the testis damage at high temperatures. Both, paternity and histological analyses showed that the 28–30 °C thermal range affects the reproductive success of *O. maya* adult males, independently of the compensatory mechanisms activated in response to the damage.

Results obtained in the present study have demonstrated that temperature is a strong environmental factor that determines the reproductive success of *O. maya*, both in laboratory and in wild populations (Angeles-Gonzalez et al., 2017; Juárez et al., 2015). In some cephalopod species studies, data demonstrate that temperature higher than experienced in wild conditions, can shorten the period of sexual maturity, reducing it by half (Takahara et al., 2016). Although this response could be apparently advantageous allowing the proliferation of cephalopods around the world (Doubleday et al., 2016), results obtained in this study evidence that in this species males and females have a temperature threshold for reproduction around 28 °C, above of which the physiological condition, the reproductive performance and success are significantly reduced.

Acknowledgments

We would like to thank CONACYT and CICESE for the scholarship granted to Laura López-Galindo; the results presented here are part of her Doctoral Dissertation at CICESE.

We thank M.C. José F. Tercero for octopus capture and laboratory conditioning; Ricardo Salomone Lopes, and Itzel Tapia for sampling.

This paper is part of the 'TempOxMar' collaboration research net (Evaluación de los efectos de la temperatura y el oxígeno disuelto en poblaciones de organismos bentónicos marinos de interés pesquero, ecológico y acuícola) organized by Universidad Nacional Autónoma de México (UNAM) and supported by Dirección General de Internacionalización-UNAM. A. Olivares is grateful for the sabbatical year (2015–2016) provided by Universidad de Antofagasta, Chile, during which this work was developed. Avila-Poveda OH is commissioned as CONACYT Research Fellow/UAS-FACIMAR (Project No. 2137), and participated as a member of the academic group 'Manejo de Recursos Pesqueros UAS-CA-132, UAS-FACIMAR' accredited to 'TempOxMar' and obtained research residency at UNAM under the

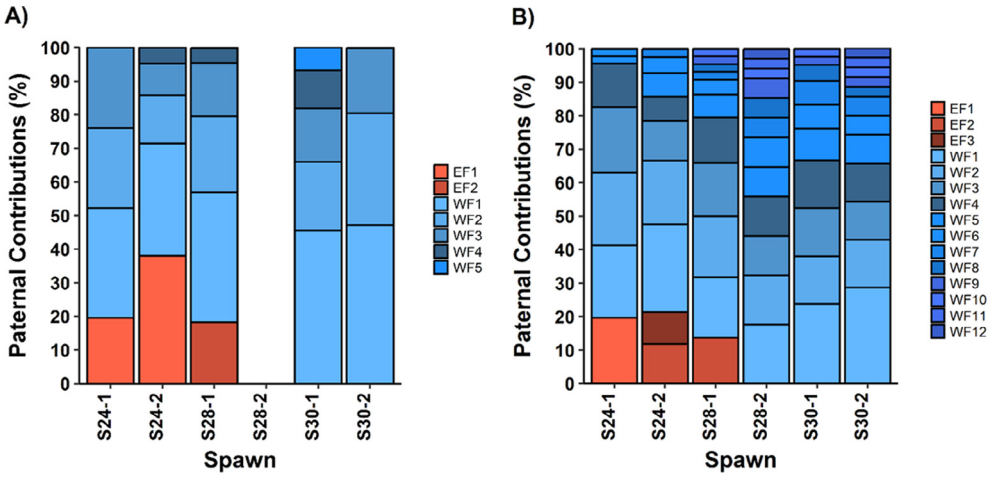

Fig. 4. Relative contributions of sires in each spawn of *Octopus maya* using GERUD (A) and COLONY (B). EF - Experimental fathers (1- first male mated; 2- Second mated and 3- Third); WF1-WF12: All unknown wild fathers.

Annual Program of Academic Cooperation UAS-UNAM (2016-NI-0036A001P001/02/03).

Competing interests

No competing interests declared.

Funding

This work was supported by the projects PAPIIT IN219116, SEP-CONACYT-CB-2014-01/241690 and CICESE: 682123.

Author contributions

L.L.G., C.G.S, C.R. designed the experiments; L.L.G., C.G.S., C.R. A.O., O.H.A.P., wrote and revised the paper; L.L.G., and C.C.M. conducted animal experimental management and care procedures; L.L.G., C.C.M., C.R., F.D. conducted physiological assessments; L.L.G., C.C.M., C.R., F.D. performed dissection, sampling and sperm quality assessments; L.L.G, C.R., A.O., O.H.A.P. performed the histological analysis; L.L.G. performed the statistical analysis; L.L.G., J.P.P., O.E.J., F.L DNA extractions; J.P.P., O.E.J., F.L. performed microsatellite amplification; L.L.G., J.P.P., O.E.J., F.L., C.G.S. Genotype assignment; L.L.G., J.P.P., O.E.J, C.G.S. Parentage analysis; C.G.S., C.R., F.L. supplied materials and supervised methodology.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2018.09.036.

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