Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California

Doctorado en Ciencias

en Ciencias de la Vida

con orientación en Biotecnología Marina

Effect of chronic thermal stress at the physiological and transcriptomic level on the performance and reproductive success of *Octopus maya* males

Tesis
para cubrir parcialmente los requisitos necesarios para obtener el grado de Doctor en Ciencias

Presenta:

Laura Liliana López Galindo

Ensenada, Baja California, México
2018

Tesis defendida por

Laura Liliana López Galindo

y aprobada por el siguiente Comité

Dra. Clara Elizabeth Galindo Sánchez
Director de tesis

Dr. Carlos Rosas Vázquez

Dr. Fernando Díaz Herrera

Dr. María Asunción Lago Lestón

Dra. Clara Elizabeth Galindo Sánchez
Coordinador del Posgrado en Ciencias de la vida

Dra. Rufina Hernández Martínez
Directora de Estudios de Posgrado

Resumen de la tesis que presenta **Laura Liliana López Galindo** como requisito parcial para la obtención del grado de Doctor en Ciencias en Ciencias de la Vida con orientación en Biotecnología Marina.

Efecto del estrés térmico crónico a nivel fisiológico y transcriptómico sobre el desempeño y éxito reproductivo de machos *Octopus maya*

Resumen aprobado por:

_____

Dra. Clara Elizabeth Galindo Sánchez
Director de tesis

Octopus maya es una especie endémica de la península de Yucatán, México. Es un organismo ectotermo particularmente sensible a la temperatura. Estudios previos en hembras de *O. maya* demostraron que temperaturas por encima de 27 °C reducen el número de huevos por desove, la tasa de fertilización y la viabilidad de los embriones. Mientras que, observaciones en machos silvestres de *O. maya* sugieren que temperaturas por debajo de 27 °C favorecen su desempeño reproductivo. Sin embargo, no se conoce si temperaturas superiores a este límite, pueden desencadenar algún efecto negativo sobre el desempeño y el éxito reproductivo. De acuerdo con lo anterior, el presente estudio tuvo como objetivo evaluar la condición fisiológica en machos de *Octopus maya*, así como el desempeño y éxito reproductivo, y finalmente, el daño histológico y el perfil de expresión transcriptómico en el testículo, bajo condiciones de estrés térmico crónico (28 °C y 30 °C), para determinar las implicaciones del calentamiento global sobre su desempeño reproductivo. Los resultados indicaron que, como se ha observado en hembras, la temperatura modula el desempeño y éxito reproductivo en machos de esta especie. Se observó que las altas temperaturas afectan negativamente el crecimiento y estado de salud de machos de *O. maya*. Los pulpos mantenidos a 30 °C presentaron un incremento en el número de hemocitos, una reducción en la tasa de consumo de oxígeno y procesos inflamatorios en el testículo. Por otro lado, no se observaron efectos en el número de espermatozoides por espermatóforo, sin embargo, hubo una mayor producción de espermatóforos en 30 °C. El análisis genético de parentesco reveló paternidad múltiple en los desoves, con un promedio de 10 machos contribuyendo a la progenie de cada desove como se ha observado en *Octopus vulgaris*, *Granelodone boreopacifica*, entre otros. Sin embargo, la contribución parental, en este caso, fue afectada por la temperatura, presentando alta, media y nula contribución en animales mantenidos a 24 °C (grupo control), 28 °C y 30 °C, respectivamente. Mediante el análisis transcriptómico del testículo se identificaron 77,661 genes y 85,249 transcritos, de los cuales 13,154 fueron anotados. El análisis de expresión diferencial del testículo reveló 1,691 transcritos involucrados en procesos biológicos de espermatogénesis, generación de gametos, desarrollo de células germinales, desarrollo y diferenciación de espermátidas, respuesta a estrés, respuesta inflamatoria, apoptosis y necroptosis. Genes como ZMYND15, KLHL10, TDRD1, TSSK2 y DNAJB13, ligados a infertilidad en otras especies, presentaron altos niveles de expresión en este estudio. En conclusión, las temperaturas de 28 °C y 30 °C afectaron la fisiología, desempeño y éxito reproductivo de machos *O. maya*, estableciendo 28 °C como un umbral térmico en los machos de ésta especie. También, se sugiere que la reducción en la fertilidad de machos expuestos a altas temperaturas, se encuentra relacionada con alteraciones en el desarrollo del espermatozoide y la motilidad.

Palabras clave: *Octopus maya*, calidad espermática, daño testicular, condición fisiológica, paternidad múltiple, reproducción, infertilidad en machos, estrés térmico crónico, RNA-Seq, inflamación, histología del testículo.

Abstract of the thesis presented **by Laura Liliana López Galindo** as a partial requirement to obtain the Doctor of Science degree in Life Sciences with orientation in Marine Biotechnology.

Effect of chronic thermal stress at the physiological and transcriptomic level on the performance and reproductive success of *Octopus maya* males

Abstract approved by:

_____

Dr. Clara Elizabeth Galindo Sánchez
Thesis Director

Octopus maya is an endemic species from the Yucatan peninsula, Mexico. It is an ectotherm organism that is particularly sensitive to temperature. Previous studies in *O. maya* females showed that temperatures above 27 °C reduce the number of eggs per spawn, the rate of fertilization and the viability of the embryos. Observations in wild males of *O. maya* suggest that temperatures below 27 °C favour their reproductive performance. However, it is not known if temperatures above this limit can trigger an adverse effect on performance and reproductive success. In agreement with the above, the present study aims to evaluate the physiological condition in males of *O. maya*, as well as the performance and reproductive success, and finally, the histological damage and the transcriptomic expression profile in the testis, under chronic thermal stress (28 °C and 30 °C), to determine the implications of global warming on their reproductive performance. The results indicated that, as has been observed in females, temperature modulates performance and reproductive success in males of this species. It was observed that high temperatures negatively affect the growth and health of *O. maya* males. Octopuses maintained at 30 °C showed an increase in the number of hemocytes, a reduction in the oxygen consumption rate and inflammatory processes in the testis. Moreover, there was a higher production of spermatophores at 30 °C and no effects were observed in the number of spermatozoa per spermatophore. The genetic analysis of kinship revealed multiple paternity in spawning, with an average of 10 males contributing to the progeny of each spawn as observed in *Octopus vulgaris* and *Granelodone boreopacifica*, among others. However, the parental contribution, in this case, was affected by temperature, presenting high, medium and no contribution in animals maintained at 24 °C (control group), 28 °C and 30 °C, respectively. The transcriptomic analysis of the testis identified a total of 77,661 genes and 85,249 transcripts, of which 13,154 were annotated. The differential expression analysis of the testis revealed 1,691 transcripts involved in biological processes of spermatogenesis, generation of gametes, development of germ cells, development and differentiation of spermatids, stress response, inflammatory response, apoptosis and necroptosis. Genes such as ZMYND15, KLHL10, TDRD1, TSSK2 and DNAJB13, linked to infertility in other species, presented high levels of expression in this study. In conclusion, temperatures of 28 °C and 30 °C affected the physiology, performance and reproductive success of *O. maya* males, establishing 28 °C as a thermal threshold in males of this species. Also, it is suggested that the reduction in fertility of males exposed to high temperatures is related to alterations in sperm development and motility.

Keywords: *Octopus maya*, sperm quality, testis damage, physiological condition, multiple paternity, reproduction, male infertility, chronic thermal stress, RNA-Seq, inflammation, testis histology.

Dedication

A mi hija Zyanya Lilian Larios López, porque todo lo que he logrado desde antes de tu llegada a mi vida siempre fue pensando en ti Toda mi vida por ti y para ti ¡Al final el logro más grande has sido tú! ¡Te amo Zyan!

A mi mami (Rafaela Romero Orozco) porque aunque ya no está físicamente conmigo, sé que me cuida, me alienta y vigila todos mis pasos. Todos los días la llevo en mi corazón señorita!!!.

A mis padres Lauro Daniel López Plauchú y María Natividad Galindo Romero por su inmenso apoyo, amor y dedicación, porque gracias a ustedes he llegado a donde me he propuesto. ¡Los amo!

Y finalmente, a mi esposo Ernesto Larios Soriano porque sin ti la vida no sería igual, porque gracias a tu apoyo y amor hemos podido cumplir nuestras metas.

Acknowledgments

Al Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California (CICESE) por permitirme realizar mis estudios de posgrado y por proveer todas las facilidades para llevar a cabo mi tesis de doctorado.

Al Consejo Nacional de Ciencia y Tecnología (CONACYT) por la beca de manutención otorgada para realizar mis estudios de posgrado. Así mismo, se agradece el financiamiento de los proyectos SEPCONACYT-CB-2014-01/241690, CICESE 682123. Así como al financiamiento parcial por la DGAPA-UNAM a través del programa PAPIIT IN219116.

A mi directora de tesis Dra. Clara E. Galindo Sánchez, por su apoyo incondicional a lo largo de mi travesía doctoral, porque siempre creíste en mí y buscaste la forma de apoyarme en todo, viajes, embarazo, conocimiento. Gracias por el apoyo a nivel personal y por todos los consejos.

A mi comité de tesis Dr. Carlos Rosas, Dr. Fernando Díaz Herrera, Dra. Asunción Lago Lestón, por su valioso apoyo y atinados comentarios que permitieron llegar hasta este momento. Gracias por el apoyo en la redacción de los artículos y tesis. Al Dr. Carlos Rosas porque siempre fue un gran apoyo, con la mejor disposición a responder mis dudas, a siempre estar pendiente del desarrollo de mi tesis y por ese gran sentido del humor en todo momento. Al Dr. Fer por su amable disponibilidad y ayuda en esos momentos en que la fisiología me jugaba chueco. A la Dra. Asunción que gracias a esas preguntas más básicas me permitió ver mis fortalezas y debilidades.

Gracias a todos los integrantes del laboratorio de genómica funcional y metagenómica Blanca, Oscar, Manuel, Edgar, Sandra, Anaid, Javier, Ricardo, por sus valiosos aportes a las presentaciones, en los labmeeting y su compañía en el laboratorio. A la Dra. Edna Sánchez, por sus sabios consejos en todo momento y por su gran ayuda en el laboratorio. A Adriana Mejía por facilitarme siempre los trámites y por su buena disposición en todo momento.

Al equipo de trabajo del Dr. Carlos Rosas en la UMDI-Sisal. A aquellos amigos que llegaron a mi vida durante mi estadía en Sisal, a Claudia Caamal, Fer Tercero, Ricardo Lopes y Juan Estefanell.

A Itzel Tapia porque una de las mejores cosas que llegaron a mi vida durante mi estancia en Sisal fue tu amistad. Porque cada día de trabajo arduo no hubiera sido lo mismo sin tu sentido del humor y apoyo. Por todos esos días que comíamos crepas y casi me aventabas el celular porque no te gustaba mi alarma. Mil gracias por todo Itz!!

A las amistades que llegaron en el momento adecuado Blanca, Giulia, Sandra porque sin ustedes estos últimos meses no hubieran sido los mismos, por todos esos momentos de charlas y chisme. Gracias chicas! A Paty por todos esos momentos de café para desahogarnos y distraernos, por tu gran amistad.

A mis padres porque gracias a ellos soy una persona de bien, que persigue y alcanza sus metas. Porque gracias a su esfuerzo y dedicación he podido lograr mis metas. Ha sido un largo y difícil camino pero lo hemos logrado!

Y por último a los grandes amores de mi vida, mi hija Zyanya Larios que llego en el momento justo para hacer mi vida más feliz, a veces más complicada pero todo ha valido la pena por ella y a mi eterno amor Ernesto Larios Soriano porque sin tu apoyo incondicional, sin tu amor y paciencia esto no sería posible. Siempre has sido un gran apoyo en mi vida y le doy gracias a la vida por habernos puesto en el mismo camino. GRACIAS AMOSHO!

Table of contents

Page

List of figures

List of tables

Chapter 1. Introduction

The Mexican four-eyed octopus *Octopus maya* is the most important species in the fishery in the Yucatan Peninsula (YP) due to the high volumes of capture. Ninety-eight percent of octopus fishery at national scale comes from the states that comprise the YP: Campeche, Yucatan and Quintana Roo, with industrial and artisanal fleets participating in it (74% of the catches in this region corresponds to *O. maya* and 26% to *O. vulgaris*). Yucatan and Campeche contribute about 90% of the national octopus fishery (Angeles-Gonzalez et al., 2017). The octopus by its capture volume is positioned in the first place of the fishing production in Mexico. However, due to its commercial value, it is positioned in the second place, and it is one of the main five national fisheries in Mexico (Arreguín-Sánchez, 2000; Hinojosa Ochoa, 2018; Juárez et al., 2015). During the period from 2001 to 2015, the average annual catch exceeded 25 thousand tons. The 69% of the catch is registered in Yucatan followed by Campeche (29%) (Hinojosa Ochoa, 2018). Thereby, *O. maya* is one of the most socioeconomically important cephalopods in the Yucatan peninsula and America (Rosas et al., 2014).

The coastal sea of the northern Yucatan Peninsula has a wide and shallow continental shelf (up to 245 km wide). It is located between the Caribbean Sea and the Gulf of Mexico, two ecosystems communicated through the Yucatan channel, which is 196 km wide and reaches 2000 m depth (Enriquez et al., 2010). In this zone the current can generate a dynamic upwelling pushing cold and nutrient-rich water uphill across the steep continental slope, reaching the Yucatan shelf where it is dispersed at the bottom (Enriquez et al., 2010). The YP is divided into two zones. The Eastern zone which is located in front of the state of Yucatan. In this zone, a seasonal upwelling allows sub-superficial subtropical water from the Caribbean (between 150 and 200 m deep) to enter the shelf at temperatures between 16 °C and 22 °C (Noyola et al., 2015). This cold water mass acts as an external temperature control for the shelf (Noyola et al., 2015). This upwelling has a very strong effect on the distribution and ecology of the organisms that inhabit the zone (Enriquez et al., 2013; Noyola et al., 2015). The western zone, located in front of the state of Campeche, does not receive the influence of the deep waters from the upwelling (Noyola et al., 2015). In the eastern zone, surficial temperatures fluctuate between 23 and 27.5 °C while in the western zone seawater-surface temperature can reach 30 °C (Figure 1) (Noyola et al., 2015; Zavala-Hidalgo et al., 2003, 2006).

The characteristics of the Yucatan continental shelf modulate the *O. maya* size (Cabrera et al., 2012), abundance, catchability (Gamboa-Álvarez et al., 2015), sexual maturation (Angeles-Gonzalez et al., 2017) and reproductive season (Avila-Poveda et al., 2016). During the fishing season (August-December), the

greatest abundances of *O. maya* are found along the coast of Campeche (Western zone), where small octopuses are fished. Meanwhile, in the eastern zone, there are less abundances, but octopuses of high biomass are captured. In the eastern zone, El Niño Southern Oscillations (ENSO) influences the productivity of the western fishery locations but not in the eastern locations (Gamboa-Álvarez et al., 2015; Juárez et al., 2018).

On the other hand, the variations in the temperature of the YP, play an important role modulating the reproduction. The reproduction of *O. maya* occurs all the year in the eastern zone whereas in the western zone brooders appear only in winter when low temperatures favour the spawn (Noyola et al., 2015). The reproduction peak of *O. maya* population occurs during the winter when temperatures in all the YP are low (Avila-Poveda et al., 2015; Noyola et al., 2015).

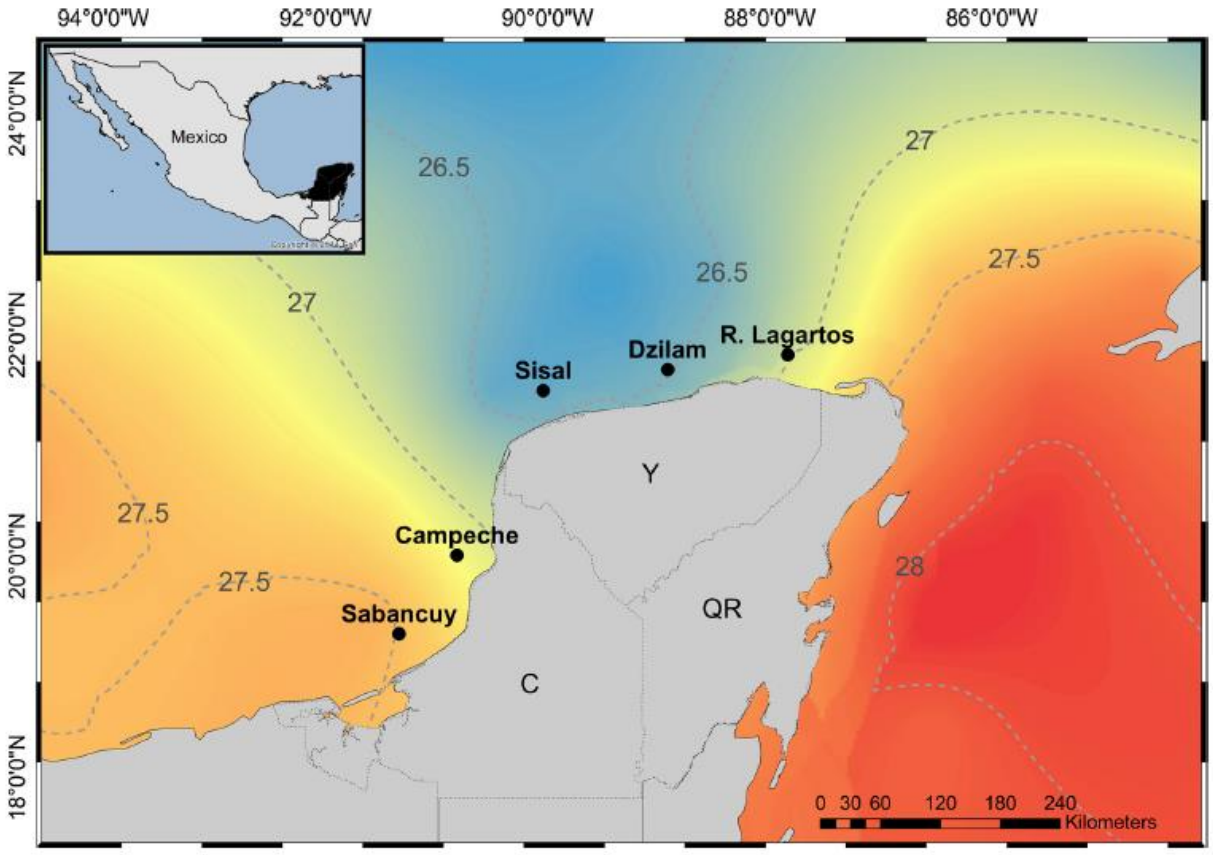

Figure 1. Mean sea temperature at 10m depth during the fishing season in the Yucatan Peninsula (Juárez et al., 2018).

1.1 Geographic distribution

Octopus maya is a benthic endemic species found on the continental shelf of the Yucatan Peninsula. It is distributed in shallow waters of the YP from Del Carmen city in the state of Campeche to Isla Mujeres in Quintana Roo (Gamboa-Álvarez et al., 2015; Noyola et al., 2013a; Rosas et al., 2014). It is a cephalopod mollusk, a carnivorous predator which diet is based primarily in crustaceans as *Callinectes similis, Libinia dubia, Melongena corona bispinosa* and *Strombus pugilis*, although it also consumes some fish (Noyola et al., 2015).

O. maya presents a life cycle between eight and twelve months (Hanlon y Forsythe, 1985), and about 9 and ten months in culture temperatures of 25 to 30 °C (Van Heukelem, 1983). The embryo development is direct, where the new hatchlings resemble an adult without present larval stages. The total embryonic development lasts between 40 to 50 days, depending on the temperature stability (Rosas et al., 2014), and between 50 and 65 days in natural environments (Solís-Ramírez, 1967).

O. maya inhabits seagrass prairies of *Thalassia testudinium*, empty shells of gastropods as *Strombus gigas*, *S. costatus* y *Pleuroploca gigantea* and is associated to coral formations and caves in the Karst plateau of the bottom at a maximum depth of 50 m, where they can find caves to spawn or for hiding of predators (Juárez et al., 2015; Solís-Ramírez, 1967).

1.2 Species description

This species is located taxonomically in the Family Octopodidae and genus *Octopus*. This species is characterized by having a soft body with a well-developed brain and eight arms that are well connected through a membrane called umbrela. Each arm has two rows of suckers; the number and disposition of suckers is an important taxonomic character. As in vertebrates, the two large and complex eyes have a crystalline lens, which provides sharp vision (Rocha, 2003; Van Heukelem, 1977). The most distinctive feature in *O. maya* is the presence of a dark spot or ocellus located beneath the eye and between the second and third arms (Voss and Solis, 1966). These organisms can mimic (change the colour and texture of skin) since their skin consists of numerous cells with pigments (chromatophores, iridophores and leucophores) responsible for the coloration and colour change (Voss and Solis, 1966). They are animals of the bottoms, so their displacement is carried out using their arms, and in case of danger they can move by

propulsion, expelling water through the respiratory cavity, which can guide in different directions (Brusca and Brusca, 2003; Rocha, 2003; Barnes, 1987).

1.3 *Octopus maya* reproduction

It is estimated that the mating of *O. maya* takes place from September where it is common to find perfectly mature females which can be determined by the naked eye, through the dorsal portion of the mantle. In November, newly deposited spawns and females incubating are observed. In December there are two conspicuous morphological regions in each egg: the embryo and the yolk sac. In January it is common to find recently hatched eggs. In February the hatching becomes more evident, ending in the normal reproductive period of the species (Solís-Ramírez, 1967).

Reproduction of animals generally depends on environmental factors, among them, light has been identified as the main factor involved in the reproductive activity of different species (Zúñiga, 1995). In *O. vulgaris* the size and age at which they reach sexual maturity seems to depend primarily on light, temperature and feeding (Mangold, 1983). The reproduction of these animals is unique; the male has the third right arm (in dorsal view) modified to introduce it into the mantle of the females, where it deposits the spermatophores (package structures that contains the sperm). The females show an annual synchronized reproductive cycle, once the female has been fertilized and finds favourable conditions, she lays the eggs on the upper surface of the shelter she chooses and stops feeding indefinitely. (Anderson et al., 2002; Mangold, 1983).

Once the spawn is placed, the female is dedicated to its protection and maintenance. She oxygenates the eggs by throwing water through the siphon and with the suction cups clean the particles that can be deposited on them. During this period the female does not leave her shelter to feed herself. To provide the energy to do this, they metabolize their own bodies. Females from the wild or captivity lost ~36-71% of their body weight, depending on the species, while brooding eggs. Although it does not interfere in the state of the offspring (Rocha, 2003; Guerra, 1992; Mangold, 1983).

After five or six weeks, the eggs hatch giving rise to juveniles with all the characteristics of an adult animal. Subsequently, the female leaves the refuge and dies within a few days, since the condition of the female deteriorates dramatically due to the loss of body weight (Van Heukelem, 1983).

1.4 The reproductive system of males *Octopus maya*

The reproductive system of *O. maya* males has a unique testis, located in the anterior part of the body, where the sperm are produced. This testis consists of several seminiferous tubules, elongated in the frontal section and semi-circular in the sagittal section (Avila-Poveda et al., 2009). The sperm exit through the vas deferens connected to a series of glands, where they are packed and surrounded by non-living transparent sheaths, giving rise to the spermatophores (Solís-Ramírez, 1967). Spermatophoric glands are responsible for the secretions that serve to agglutinate the sperm, as well as the formation of the membranes that surround them (Rocha, 2003).

Fully formed spermatophores are stored in the Needham's sac (storage sac) from which they exit through the seminal afferent sac and from the terminal organ (penis), until copulation (Wodinsky, 2008; Rocha, 2003). To initiate the transfer of sperm a spermatophore stored in the penile diverticulum is driven into a groove in the ventral margin of the hectocotylus. The hectocotylus is usually the third right arm that is shorter than the third left arm and does not have suckers on the tip, forms a end organ that serves as an insemination organ (Wodinsky, 2008; Voight, 2002). This organ consists of the ligule and the calamus that is presumed to position the spermatophore in the oviduct of the females before releasing the sperm (Wodinsky, 2008; Voight, 2002).

The membrane along the third right arm is modified to form a groove that transfers the spermatophore to the female. This groove, formed simply by folding the muscle to form a tube, runs from the interbranchial membrane midway between the third and fourth right arms along the third right arm ending in the calamus (Wodinsky, 2008).

The spermatophores exits the Needham's sac and the penis initially with its posterior ends first. This means that the spermatophore must be reversed so that the ejaculatory (anterior, oral, cap) end faces the female’s oviduct, since it is from this end that the sperm emerge during the spermatophoric reaction, which starts when the spermatophore is removed from the Needham's sac by the hectocotylus (Hoving et al., 2010). The Needham's sac has a limited storage space, so the spermatophores are constantly eliminated to make room for the new spermatophores (Voight and Feldheim, 2009).

The movements of the hectocotylus, the inter-branchial membrane, the siphon, the groove, the mantle musculature and the penis are involved in the transfer of the spermatophore (Wodinsky, 2008). Wodinsky (2008) showed that the siphon does not play a role in the inversion of the spermatophore or the placement

of the spermatophore in the furrow. The siphon serves as a tube or conduit through which the penis invests and transfers the spermatophore.

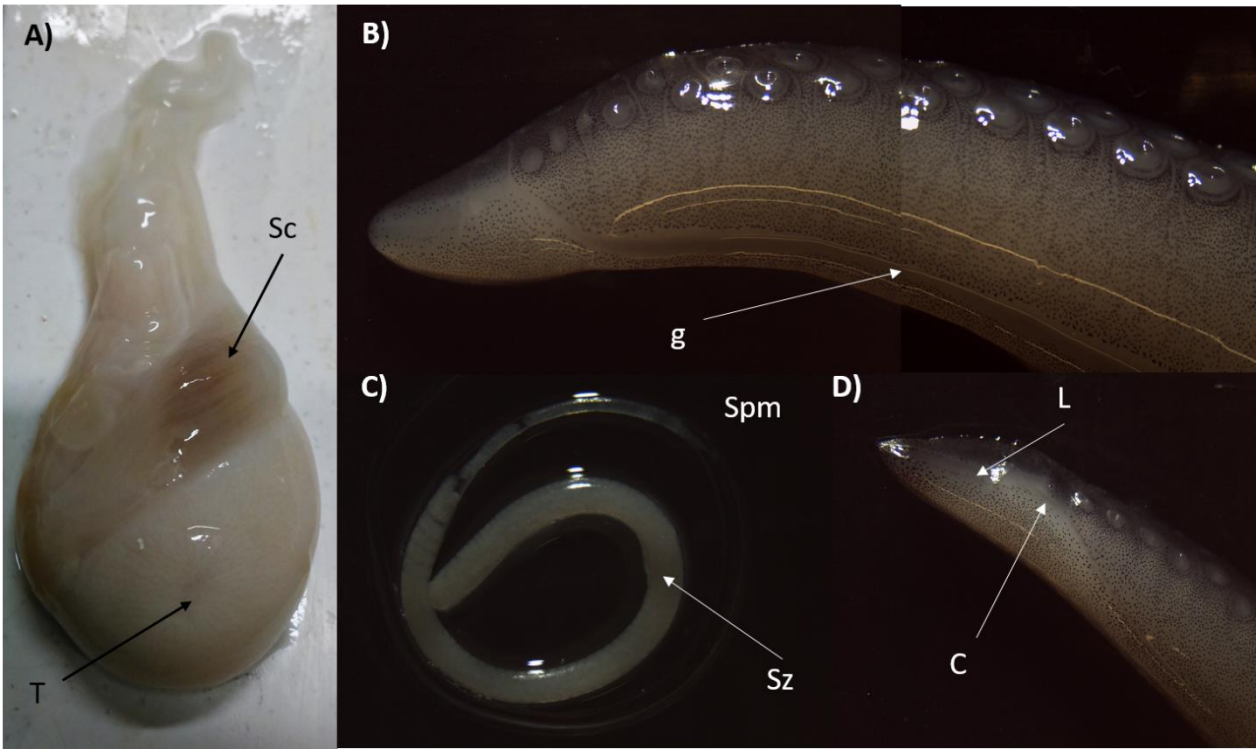

Figure 2. Reproductive system of *O. maya* males. A) Gonad of *O. maya* males constituted for the spermatophoric complex (Sc) and the testis (T). B) Hectocotylus of *O. maya* showing the groove where spermatophores are transferred. C) Spermatophore (Spm) of *O. maya* where the sperm mass can be observed. D) Tip of the hectocotylus showing the Ligula (L) and Calamus (Source: Personal collection).

1.4.1 Transcriptomic analysis in cephalopods

In general, there are little transcriptomic information in cephalopods and it is mainly focused on: the central nervous system and the response to infection of the common octopus *O. vulgaris* (Castellanos-Martínez et al., 2014; Zhang et al., 2012), eye development of *Nautilus* and pigmy squid (Sousounis et al., 2013), immune and hematopoietic system of the squid *Euprymna tasmanica* (Salazar et al., 2015), secreted slime *Sepiadarium austrinum* (Caruana et al., 2016), embryonic development in the cuttlefish *Sepia suculenta* (Liu et al., 2016), sexual precocity of the cuttlefish *Sepiella maindroni* (Tian et al., 2018), morphological differences in dimorphic euspermatozoa of the squid *Loligo bleekeri* (Yoshida et al., 2014) and gonadal development of the common Chinese cuttlefish *Sepiella japonica* (Lü et al., 2016).

Yoshida et al., (2014) perform a transcriptomic and proteomic analysis of the testes of consort and sneaker males to search for differences in testicular mRNAs and sperm proteins. They found a family encoding dynein heavy chain gene and many enzymes involving energy metabolism as phosphoprotein phosphatase 1 (PP1).

1.5 Spermatogenesis and spermiogenesis processes in the testis

Spermatogenesis is a dynamic, highly-regulated and synchronized process of maturation of stem spermatogonia into mature spermatozoa, which take place in the seminiferous tubules in the testis (Shaha et al., 2010). This process involves the mitotic development of spermatogonia and their differentiation into spermatocytes followed by the formation of spermatids and mature spermatozoa (Shaha et al., 2010). The spermatogenesis process consists of several phases: a) mitosis proliferation where spermatogonial stem cells are divided to produce spermatocytes; b) meiotic phase which implies the spermatocyte division to produce haploid round-spermatids; and c) spermiogenesis to produce mature elongated-spermatids (Akmal et al., 2016).

Spermiogenesis is the process by which haploid round spermatids complete an extraordinary series of events to become streamlined spermatozoa capable of motility (O'Donnell, 2014). Spermiogenesis begins after spermatocytes complete two quick successive meiotic reductive divisions to produce haploid round spermatids (Dang et al., 2012; He et al., 2012; O'Donnell, 2014; Yan et al., 2010). The different phases of spermiogenesis are distinguished by the morphological appearance of the developing acrosome and the changing shape of the nucleus (O'Donnell, 2014). The first phase involves the nucleus condensation and the acrosome formation (Yan et al., 2010). As the spermatid nucleus compacts, nucleosomal chromatin is transformed into compacted chromatin fibers by the replacement of histones with transition proteins, which are subsequently replaced by protamines (important role in male's normal fertility) (Akmal et al., 2016). The spermatid ceases active gene transcription as nucleosomes disappear and the chromatin is remodelled and compacted (O'Donnell, 2014). Accordingly, earlier round spermatids transcribe many mRNAs that are necessary for spermiogenesis, however many of those mRNAs are subjected to translational delays until the protein is required later in spermiogenesis (O'Donnell, 2014; Yan et al., 2010). The second phase of the spermiogenesis involves the assembly of the sperm flagellum, where the central component is the microtubule-based axoneme. It is assembled soon after the completion of meiosis. As spermatids elongate, the accessory structures needed for flagella function (dense outer fibers, fibrous

sheath, mitochondrial sheath) are assembled around the central axoneme (O'Donnell, 2014). The final phase is the spermiation where the elongated spermatids undergo their final remodelling and release from the seminiferous epithelium. The spermiation is a complex multi-step process, which is highly vulnerable to disruption (O'Donnell, 2014). The major morphological defects observed under infertility conditions are abnormal acrosome development, abnormal sperm head shape, abnormal sperm tail development, disordered spermatid orientation and disruptions to spermiation (Li and Liu, 2014; Lo et al., 2012; O'Donnell, 2014).

1.6 Multiple paternity

Cephalopods are typically highly promiscuous, and females of most species store sperm from multiple males, and for long periods of time (Birkhead and Møller, 1998; Squires et al., 2014). As a consequence, multiple paternity has been identified in different cephalopod species. For example, *O. olivery* has two oviducts for sperm storage and may be able to store viable sperm for at least 100 days and up to ten months (Ylitalo-ward, 2014). A small number of studies have investigated multiple paternity within cephalopods. Voight and Feldheim (2009) sampled young deep-sea octopus, *Graneledone boreopacifica*, and found at least two genetically distinct sires contributed to the hatchlings analyzed. Quinteiro et al. (2011) sampled egg clutches of the common octopus, *O. vulgaris*, and found evidence of between two to four males contributing to each clutch. Squires et al. (2011) also found evidence of multiple paternity in dumpling squid, *Euprymna tasmanica*, egg clutches. Finally, Larson et al., (2015) collected several egg strings of the giant Pacific octopus, *Enteroctopus dofleini* and found strong evidence of multiple paternity within one egg clutch with progeny sired by between two to four males.

1.7 Effects of the temperature on the *Octopus maya* reproduction

The effect on the reproductive traits in females of *O. maya* has been widely studied. Juárez et al., (2015) maintained female *O. maya* at two constant temperatures (24 °C and 31 °C) and a thermal ramp (From 31 °C to 24 °C with a decreasing rate of 1 °C/5d) and then evaluate the number of spawns, eggs spawned by female, fertilization percentage, embryos development, and hatchlings survival after starvation for each thermal regime. Their results indicated that high temperatures (31 °C) inhibit spawning and only 13% of females spawned. The few fertilized eggs spawned died after two weeks. Females exposed to temperature

changes only spawned after temperature reached below 27 °C where 87% of the females spawned. Juárez et al., (2015) establish a thermal threshold of 27 °C for female *O. maya* where temperatures below this, favour and stimulate spawning, fertilization, embryonic development and survival of hatchlings. In the same way, Juárez et al., (2016) observed that hatchlings from stress *O. maya* females were smaller and had a lower growth rate compared to those from unstressed females providing evidence that temperature stress experienced by females has consequences on the performance of hatchlings, with effects on the biomass production and survival.

Caamal-Monsreal et al., (2016) observed that embryos maintained at high temperatures (30 °C) presented low weight and size, a high proportion of yolk remaining in the eggs (60% of the initial yolk volume) and a depressed metabolic rate, indicating that such high temperature can cause serious physiological alterations in embryo development processes, producing premature hatchlings. The rate of hatching was dramatically reduced at 30 °C with only 31% of the embryos hatched after 30 d of incubation. When those hatchlings were maintained for 10 d without food, 37% of them died, indicating that the general health condition of hatchlings, together with mechanisms related to the use of reserves, were seriously affected. Finally, they established that temperatures between 22–24 °C is optimal for embryo development with a limit thermal threshold at 26 °C.

1.8 Justification

The octopus *O. maya* is a species highly susceptible to environmental changes, mainly because its distribution is restricted to the Yucatan shelf. It has been observed that temperature is a key factor in reproduction. However, little is known about the molecular mechanisms involved in the regulation of sperm production and quality in males, and the effect that temperature has on these. Due to the above, it is proposed to conduct a study on the impact of temperature on the physiological performance and reproductive success of *O. maya* males. Additionally, we perform a transcriptomic analysis to elucidate the molecular mechanisms involved in the regulation of spermatogenesis in the testis under chronic thermal stress and co-relate it at the same time with the sperm quality, survival of their offspring and parental contribution.

1.9 Hypothesis

Chronic thermal stress will cause alterations in physiology, testicular structure, performance and reproductive success in *Octopus maya* males. Likewise, temperature and reproductive activity will induce a change in the expression profiles and processes involved in the stress response and spermatogenesis in the testis of *O. maya*.

1.10 Objectives

1.10.1 General objective

To evaluate the effect of the chronic thermal stress over the reproduction and the transcriptomic profile of the testis in *Octopus maya* males sexually matures.

1.10.2 Specific objectives

- To evaluate the effect of chronic thermal stress on the physiology of *Octopus maya* males.
- To evaluate the reproductive performance trough reproductive indexes, seminal quality and the histology of the *O. maya* testis.
- To determine the effect of the paternal thermal stress in the reproductive success of *O. maya* through the mating with non-stressed females.
- To evaluate the effect of the temperature on the genes that regulate the reproduction and stress response in *O. maya* males subjected to chronic thermal stress through a massive sequencing analysis RNA-Seq.
- To evaluate the effect of temperature on the expression of the genes involved in reproduction and the production of spermatozoa in adult males of *O. maya,* through the analysis of RNA-Seq.
- To evaluate the levels of expression of genes involved in spermatogenesis processes, response to stress and inflammation through quantitative analysis in real-time PCR.

Chapter 2. Reproductive performance of *Octopus maya* males is conditioned by thermal stress.

2.1 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 °C-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 the Yucatán Peninsula, located at the entrance of Gulf of Mexico

2.2 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 strongly influenced by global warming (Nguyen et al., 2011). For example, the 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 function as an external temperature control for the shelf, and 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 Yucatan Peninsula (YP) continental shelf and the most important octopus fishery in the American continent, with an annual production fluctuating between 8,000 and 20,000 Tons (Galindo-Cortés et al., 2014; Gamboa-Álvarez et al., 2015; SAGARPA, 2013). *O. maya* as an ectotherm organism is particularly temperature-sensitive (Noyola et al., 2013a, 2013b) that can be affected in its morphology, behaviour, physiology and reproduction by changes in ambient temperature with spatio-temporal fluctuations. Predictions of the thermal processes on the YP shelf indicate that sea temperatures may rise between 2.5 to 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 octopuses were fished; whereas, in the eastern zone, fewer 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 hatchlings 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 et al. (2017) postulated the hypothesis that thermal condition causes migration of octopuses from western to eastern zone of the 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 °C to 25 °C**)**. Juárez et al., (2018) analyzed 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. Their results showed that there is significant genic differentiation in the *O. maya* population that match with the two different thermal zones where *O. maya* is distributed (Juárez et al., 2018). This suggests 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 *Granelodone 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 the 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 hemocytes 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.

2.3 Material and methods

2.3.1 Ethics Statement

In this study, octopuses were anesthetized with ethanol 3% in seawater at experimental temperatures (Estefanell et al., 2011; Gleadall, 2013) to induce narcotization to enable humane killing (Andrews et al., 2013) in consideration of ethical protocols (Mather and Anderson, 2007), and the animal´s 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.

2.3.2 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 $\pm$ 1 °C). The ponds were covered with black mesh reducing direct sunlight to 70%, and connected to seawater recirculation systems coupled with protein skimmers and 50 µmb bag filters. PVC 50 mm diameter open tubes were offered as refugees in proportion 2:1 per animal. Octopuses were fed twice a day individually with a paste made with squid and crab meat at a ratio of 8% of its body weight (Tercero et al., 2015).

2.3.3 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 1,800-Watt heaters connected to automatic temperature controllers, while the temperature of 24 °C was controlled with a titanium chiller and the air conditioning of the experimental room.

2.3.4 Physiological condition

2.3.4.1 Specific growth rate and digestive gland index

We used 23 octopus adult males to evaluate the 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 seawater 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 to 150 µL was drawn using a catheter inserted in the dorsal aorta. The sample was kept in ice until the hemocytes count. Once samples were obtained, octopus was 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 (Valverde et al., 2008).

2.3.4.2 Total hemocytes count and hemocyanin concentration (Hc)

Total hemocytes 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.

2.3.4.3 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.

2.3.4.4 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 know 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).

2.3.5 Reproductive performance

2.3.5.1 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 Needham´s 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.

2.3.5.2 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 400x 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 1^{st} and 2^{nd} order, round spermatid, ovoid spermatid, and elongated spermatid), height of proliferative stratum (PS: spermatogonia, spermatocytes of 1^{st} and 2^{nd} order, and round spermatid), and height of differentiation stratum (DS: ovoid spermatid and elongated spermatid), where SGC = PS + DS (Supplementary Figure 19). 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.

2.3.6 Male Reproductive success

2.3.6.1 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 *O. maya* females were maintained in 80L 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 each experimental temperature (Figure 3). 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.

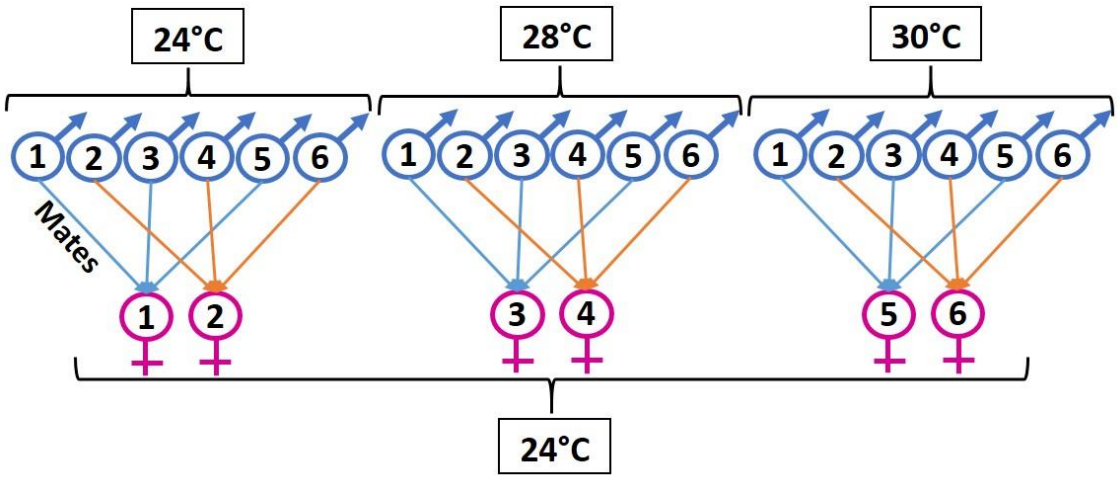

Figure 3. Mating system used in *Octopus maya* per experimental temperature. Males maintained at different experimental temperatures (24 °C, 28 °C and 30 °C) during 30 d were mated with females at 24 °C. The matings were done one by one for each temperature. Copulation lasted 4 to 6 hours and between 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.

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).

2.3.7 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 homoscedasticity 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.

2.3.8 Paternity analyses

2.3.8.1 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 was 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 electrophoresis in agarose gel (1%) at 85V for 40 min.

2.3.8.2 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 85V for 40 min. The amplicons marked with different fluorophores obtained from the same sample were multiplexed for fragment analysis in an AB genetic analyzer (Applied Biosystems).

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.

2.3.8.3 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.

2.3.8.4 Parentage and Data Analyses

The paternity analyses were conducted using two different software 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-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 (Supplementary Table 9) were obtained with the ARLEQUIN software.

2.4 Results

2.4.1 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 nine times higher than the observed in octopuses maintained at 30 °C. In consequence, an SGR six 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 ($2.5x10^6 \pm 1.5x10^6$ 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 $\pm$ 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; $P < 0.05$).

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

Parameters	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	$1.5x10^{6} \pm 7.9x10^{5}$ ^{a}	$2.2x10^{6} \pm 8.2x10^{5}$ ^{a}	$2.5x10^{6} \pm 1.5x10^{6}$ ^{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); VO2, Oxygen consumption (mgO2 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.

Parameters	Temperature			Statistics
	24 °C	28 °C	30 °C	ANOVA
N	23	23	23	-
TSC	$1.3x10^{6} \pm 5.7x10^{5}$ ^{a}	$1.4x10^{6} \pm 4.7x10^{5}$ ^{a}	$1.4x10^{6} \pm 3.5x10^{5}$ ^{a}	n.s.
TASC	$5.2x10^{5} \pm 2.3x10^{5}$ ^{a}	$5.5x10^{5} \pm 1.9x10^{5}$ ^{a}	$5.3x10^{5} \pm 1.3x10^{5}$ ^{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 (%).

2.4.2 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, dilation of the seminiferous tubules and their lumen were evident, from 24 °C to 28 °C increasing 50-60 microns, while from 28 °C to 30 °C the dilation increased another 80-100 microns. Despite the expansion of the seminiferous tubules and lumen, each one 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 microns 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% to 5%, except octopuses treated at 30 °C, which showed a 4-fold of these completely acidophilic bodies compared to the other treatments (Figure 4). At 30 °C, we observed acidophilic bodies with basophilic material, and vacuolated basal compartments (Figure 5).

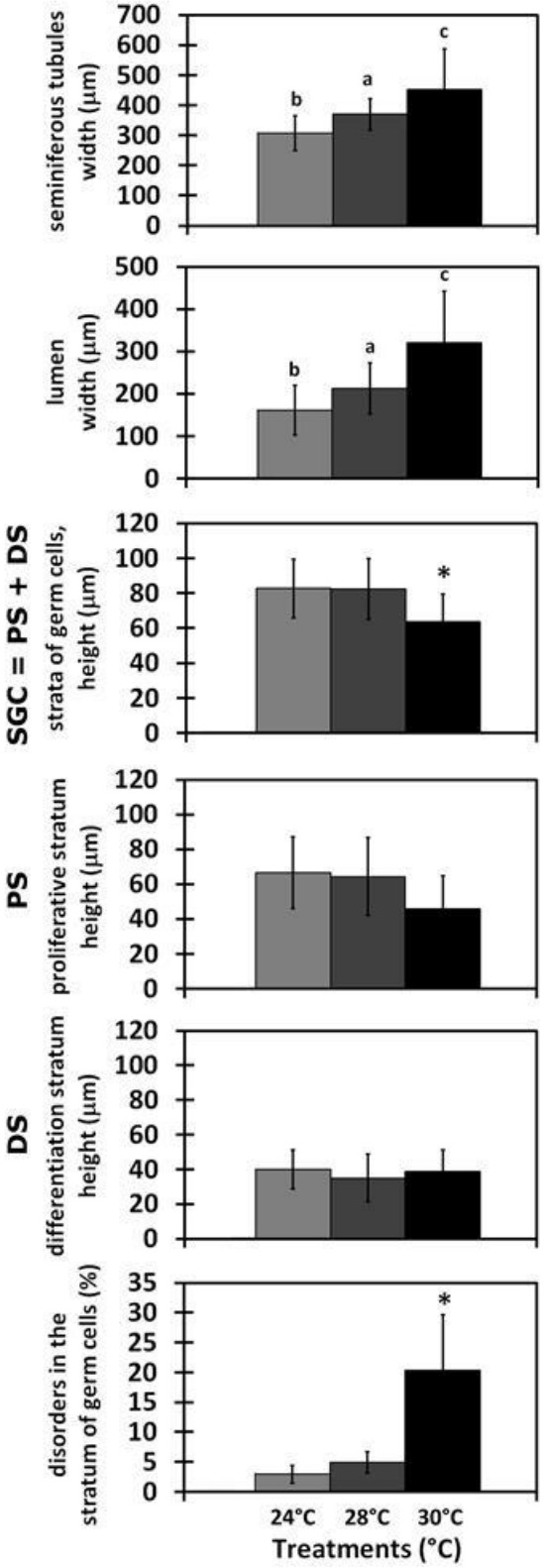

Figure 4. Morphological changes in the germ cells strata (SGC = PS + DS), and the seminiferous tubules lumen during experimental thermal stress. Values are mean ± SD. Different letters indicate significant differences among treatments and asterisks denote significant differences from all other treatments at $P < 0.05$.

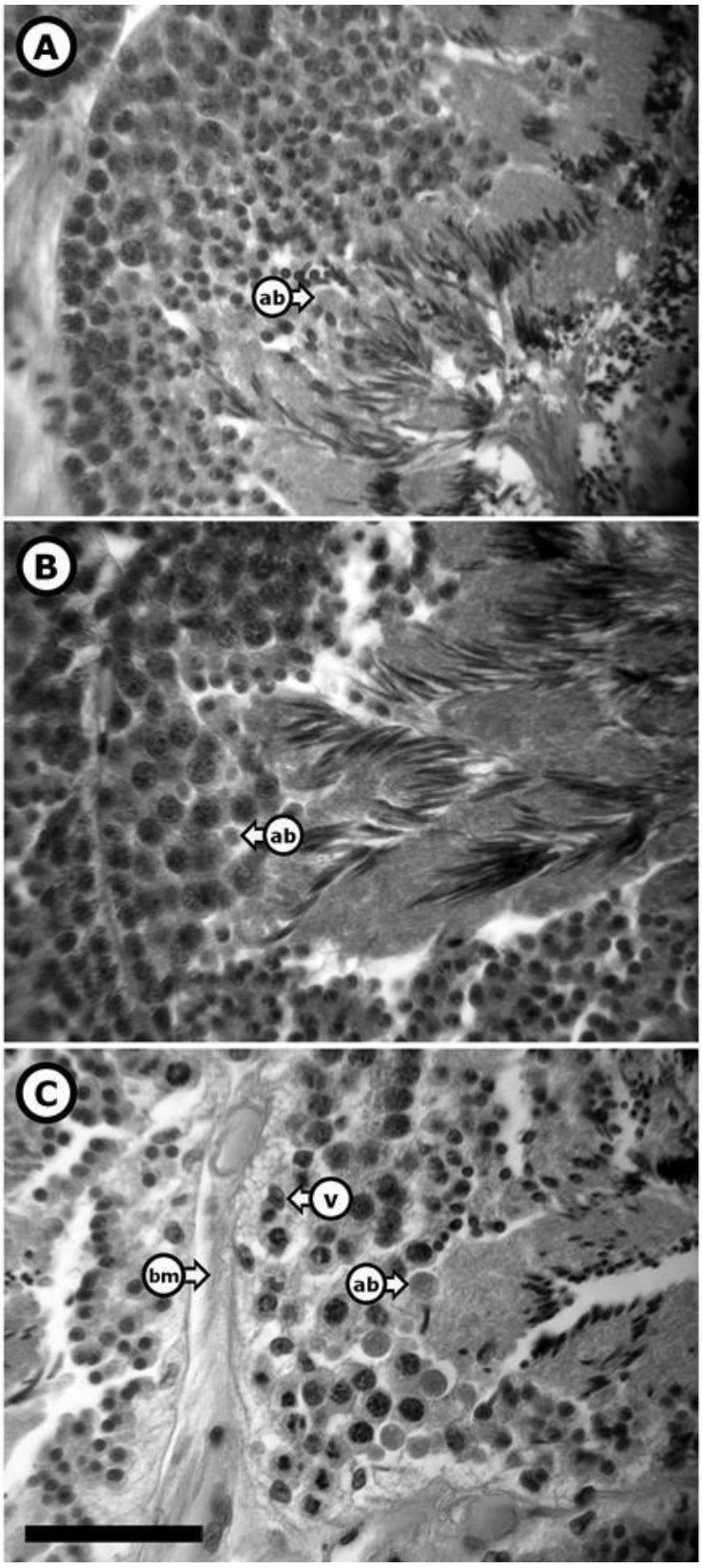

Figure 5. Cross sections photomicrographs of *Octopus maya* seminiferous tubules during the chronic thermal stress. Treatments are: A) 24 °C, B) 28 °C and C) 30 °C. ab- acidophilic bodies, bm-basement membrane of the seminiferous tubule, v- vacuole in the basal area. General structure followed the scheme from Supplementary Figure 19. Scale bars are 50 µm.

2.4.3 Male reproductive success

2.4.3.1 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.

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.

2.4.4 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-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-E}. In the case of Omy4-18 were within H_{W-E} in B and O (Table 5).

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

Locus	N_a	H_o	H_e	P_{H-W}	F_{IS}
Omv2-0					
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-W}, P-values for Hardy-Weinberg equilibrium; F_{IS}, Inbreeding coefficient . *P < 0.01; **P < 0.001.

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, Figure 6A). 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, Figure 6A); 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, Figure 6A). 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, Figure 6A).

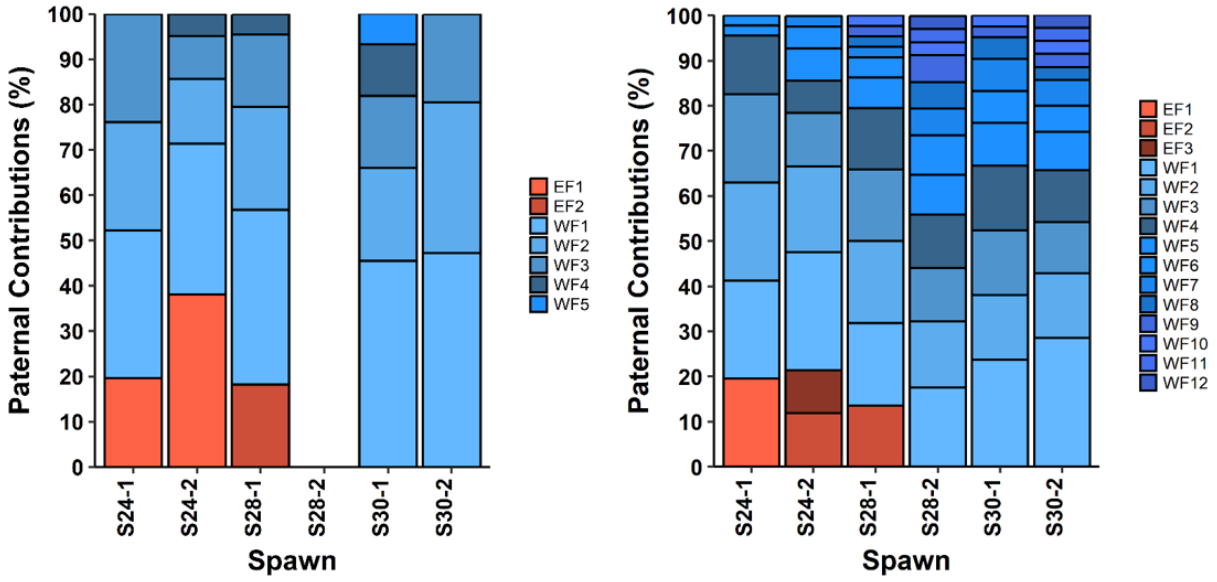

Figure 6. Relative contributions of sires in each spawn of *Octopus maya* using GERUD (A) and COLONY (B). EF - Experimental fathers (1- first male mated; 2- Second mated and 3- Third); WF1-WF12: All unknown wild fathers.

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, Figure 6B). 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, Figure 6B). 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 (Figure 6B).

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.

2.5 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 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., 2015, 2016; Sanchez-García et al., 2017). Results obtained in the present study, demonstrate that temperature of 30°C

affected growth rate negatively. 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 hemocytes, 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 the 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 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 and spermatozoa with some damage, inferred through the increase of acidophilic bodies observed in testis. These apoptotic bodies and the inflammatory process are characteristics of necroptotic processes and infertility (Lin et al., 1997; Shaha et al., 2010). There is at least three hypothesis that could be postulated to explain why the increase in spermatophores and spermatozoids in animals exposed at 30 °C did not contribute to impregnate females:

1) When impregned, spermatozoids cannot reach the oviductal gland due to motility limitations derived of some type of damage on the energy pathway in the cell and/or damage to the mitochondria that conforms the sperm tails; 2) There is a kind of damage in the spermatozoids that provoked damaged 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. 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 24.7% 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 °C-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.

Chapter 3. Transcriptomic analysis reveals insights on male infertility in *Octopus maya* under chronic thermal stress.

3.1 Abstract

Octopus maya endemic to the Yucatan peninsula, Mexico, is an ectotherm organism particularly temperature-sensitive. Studies in *O. maya* females show that temperatures above 27 °C reduce the number of eggs per spawn, fertilization rate and the viability of embryos. High temperatures also reduce male reproductive performance and success. However, the molecular mechanisms are still unknown. The transcriptomic profiles of testes from thermally stressed (30 °C) and not stressed (24 °C) adult male octopuses were compared, before and after mating to understand the molecular bases involved in the low reproductive performance at high temperature. The testis paired-end cDNA libraries were sequenced using the Illumina MiSeq platform. Then, the transcriptome was assembled *de novo* using Trinity software. A total of 53,214,611 high-quality paired reads were used to reconstruct 85,249 transcripts and 77,661 unigenes with an N50 of 889 bp length. Later, 13,154 transcripts were annotated implementing Blastx searches in the UniProt database. Differential expression analysis revealed 1,691 transcripts with significant difference among treatments. Functional annotation and pathway mapping of differential expressed genes revealed significant enrichment for biological processes involved in spermatogenesis, gamete generation, germ cell development, spermatid development and differentiation, response to stress, inflammatory response and apoptosis. Remarkably, genes such as ZMYND15, KLHL10, TDRD1, TSSK2 and DNAJB13, which are linked to male infertility in other species, were differentially expressed among the treatments. The expression levels of these key genes, involved in sperm motility and spermatogenesis were validated by quantitative real-time PCR. The results suggest that the reduction in male fertility at high temperature can be related to alterations in spermatozoa development and motility.

3.2 Introduction

Octopus maya endemic to the Yucatan Peninsula (YP) is an ectotherm organism particularly temperature-sensitive mainly due to the characteristics of its habitat (Noyola et al., 2013a, 2013b, 2015). It is one of the most important commercial fisheries in the YP and the American continent (Gamboa-Álvarez et al., 2015). The YP is divided into two distinct zones. The eastern zone located in front of the Yucatan state presents a summer upwelling that brings a mass of cold water from the Caribbean (16 °C-22 °C) that enters the YP

and acts as an external temperature control for the shelf with temperatures fluctuating between 23 °C to 27.5 °C (Noyola et al., 2013b). Meanwhile, the western zone located in front of Campeche has no influences of deep cold waters, and as a consequence, the surficial temperatures can rise above 30 °C in summer (Noyola et al., 2013b, 2015). Gamboa et al., (2015) observed that temperature modules two essential aspects in the YP such as the fishing seasons which presents higher octopus abundances with low biomass in the western zone. In the eastern zone, lower abundances and higher biomass had been recorded. In the other hand, low temperatures in the different zones of the YP favour the spawning (Avila-Poveda et al., 2015; Gamboa-Álvarez et al., 2015; Noyola et al., 2015).

Temperature plays a crucial role in different life aspects of *O. maya*. In females, it has been observed that temperatures above 27 °C inhibit the spawning and drastically reduce the eggs production, the fertilization rate, the embryonic development time, the number of hatchlings and hatchling survival (Juárez et al., 2015, 2016). In embryos, high temperatures increase the metabolic rates affecting the embryo development (smaller embryos) and hatching rate. Embryos have a thermal threshold at 26 °C and temperatures around 30 °C inhibit growth, reduce the metabolic rate and embryos present a high yolk proportion (Caamal-Monsreal et al., 2016; Sanchez-García et al., 2017). Recently, in males of *O. tankahkeei*, through histological analysis in the testis of octopus exposed to 32 °C for two h, Long et al., (2015) observed ultrastructural changes and damaged mitochondria in spermatocytes and spermatids.

The testis is the male gonad, responsible for the production of male gametes via spermatogenesis and androgenic hormones (Waiho et al., 2017). The spermatogenesis is a dynamic, synchronized and highly regulated process that involves the division and differentiation of spermatogonial germ cells into mature spermatozoa, which take place in the seminiferous tubules (Akmal et al., 2016; Shaha et al., 2010). The normal process begins with a spermatogenic phase regulated by mitotic divisions, followed by two meiotic divisions to produce secondary spermatocytes and ends with spermiogenesis, a remarkably morphological transformation process. The spermiogenesis involves: a) nucleus condensation, where DNA is compacted by protamines; b) formation of acrosome that contains hydrolytic enzymes crucial for oocyte penetration during fertilization; c) flagellum formation, which involves the development of microtubules arising from the centrioles of the round spermatid; and d) cytoplasm reorganization, where a large part of the cytoplasm is phagocytosed by the Sertoli cells, that constitutes a hematotesticular barrier (Sheng et al., 2014).

The morphology and ultrastructure of testis and germ cells in *O. maya* and their histological changes during sexual maturation has been described in detail by Avila-Poveda et al., (2009, 2016). In a previous work of

our team, the effect of thermal stress over the physiology and the reproductive performance and success of male *O. maya* exposed to preferred (24 °C) and stress (28 °C and 30 °C) temperatures was assessed. Our research findings indicated that chronic thermal stress inhibited the growth rate: organisms exposed to 30 °C had a specific growth rate six times lower than those exposed to 24 °C and gained weight nine times lower. A significant reduction in oxygen consumption was identified with increasing temperatures. High temperatures induced the immune response in *O. maya* males by increasing the circulating hemocytes in the hemolymph. At the reproductive level, a significant increment in the production of spermatophores with increasing temperatures was observed. Although, despite this reproductive strategy, the reproductive success was affected, with no parental contribution from octopus exposed to 30 °C. The histological analysis of the testis showed damage from moderate to severe in octopus exposed to 28 °C and 30 °C, seriously affecting the cellular testis organization (López-Galindo et al., 2019).

Nevertheless, the molecular mechanisms that regulate reproduction process in *O. maya* males and the response to environmental factors as temperature are poorly understood. The transcriptome analysis through RNA-Seq methodology could reveal genes that are being actively expressed in testis of *O. maya* under chronic thermal stress and facilitate the discovery of novel genes involved in this response and the reproductive processes with high sensitivity and accuracy as has been successfully identified in other invertebrate species as the Pacific oyster *Crassostrea gigas* (Kim et al., 2017; Lim et al., 2016), green lip abalone *Haliotis laevigata* (Shiel et al., 2014), snail *Echinolittorina malaccana* (Wang et al., 2014), king scallop *Pecten maximus* (Artigaud et al., 2015), Chinese mitten crab *Eriocheir sinensis* (Li and Qian, 2017), orange mud crab *Scylla olivacea* (Waiho et al., 2017) and squid *Loligo bleekeri* (Yoshida et al., 2014). To date, in cephalopods transcriptome information related to reproduction is still insufficient. In this regard, this study aims to provide insights into the molecular mechanisms that regulate the reproductive processes such as spermatogenesis and spermiogenesis in testis of *O. maya* under chronic thermal stress. Here, we present a comprehensive analysis of the transcriptome data obtained from testis tissue of *O. maya* males exposed to optimal, intermediate and stressful temperature before and after mating using Illumina Miseq. This study is the first report of how octopus male fertility is affected at the molecular level and which mechanisms are triggered to cope with chronic thermal stress. Our results indicated that despite the adaptative mechanisms present in *O. maya* to tolerate temperatures close to 30 °C, apparently a prolonged exposure to them causes infertility related to alterations in sperm development and motility.

3.3 Material and methods

3.3.1 Ethics statements

We established protocols that 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). Octopuses were anaesthetized with 3% ethanol to induce narcotization to enable humane killing in consideration of animal's welfare during manipulations (Andrews et al., 2013; Estefanell et al., 2011; Mather and Anderson, 2007).

3.3.2 Experimental design and sampling

O. maya males were captured off the coast of Sisal Yucatan, from June to September of 2015, we obtained a total of sixty-three testis samples. Thirty-six testis were sampled from males before the copula (PRE) that were maintained in 80 L individual tanks and exposed at three experimental temperatures during 30 days (n=12 per treatment): a) preferred temperature (24 °C; 24PRE); b) intermediate temperature (28 °C; 28PRE); and c) stress temperature (30 °C; 30PRE). Meanwhile, twenty-seven testes were sampled from males after the copula (POS), exposed to chronic thermal stress and mated with females maintained at 24 °C (n=9 per temperature). Testis samples were removed surgically and immediately preserved in Nap buffer (Camacho-Sanchez et al., 2013), and stored at -80 °C until required. Furthers details are shown in López-Galindo et al., (2019).

3.3.3 RNA isolation, library preparation and sequencing

Total RNA was obtained from 30 mg of testis tissue homogenized in Fastprep-24 Instrument (MP Biomedicals, Solon, OH) with a speed of 5.0 m/s for 20 s. Then, total RNA was extracted using the RNEasy Plus mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Total RNA samples were then digested with RQ1 RNase-Free DNase (Promega, Madison, WI) to remove potential genomic DNA contamination using the manufacturer's protocol with an additional precipitation and purification steps as follows: each treated sample was precipitated with 1:10 volumes of 3M sodium acetate and 3 volumes

of absolute Ethanol at -80 °C for 1 h; centrifuged at 13,000 rpm for 10 min at 4 °C. The RNA pellets were washed with 200 µl of cold 70% Ethanol; centrifuged at 7,500 rpm for 10 min at 4 °C and dried at room temperature for 10 min. The RNA pellets were resuspended in RNase free-DNase water. The quality of the RNA was assessed by 1% agarose gel electrophoresis and quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). For transcriptomic analysis, only the organisms exposed to 24 °C and 30 °C were sequenced. To construct the libraries, we prepared three different pools with equal amounts of RNA from four individuals per experimental condition (24PRE and 30PRE, 24POST and 30POST).

The quality of the 12 RNA pools (three pools 24PRE, three pools 24POST, three pools 30PRE and three pools 30POST) were analyzed with an Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). cDNA libraries were prepared using the TruSeq®RNA Sample Prep kit V2 (Illumina, San Diego, CA) following manufacturer's protocol. Amplified libraries were purified with AMPure XP magnetic beads (Beckman Coulter, Brea, CA). The fragment sizes were verified and quantified with the 2100 Bioanalyzer system. The 12 paired-end libraries were normalized at 4 nM and then pooled equally. They were sequenced using the MiSeq Reagent Kit v3, with a read length of 2 x 75 bp on Illumina MiSeq sequencing system (San Diego, CA, USA). PhiX control was used at 1% for cluster generation.

3.3.4 *De novo* transcriptome assembly

The FastQC software was used to assess the quality of the raw reads (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Then, Illumina adapters, indexes and low-quality reads were removed with Trimmomatic version 0.36 (Andrews et al., 2013; Bolger et al., 2014). Clean reads with a Phred33 score > 30 and length > 36 bp were used in subsequent analysis. The testis reference transcriptome was assembled *de novo* (including all the libraries) using Trinity version 2.4.0 (Grabherr et al., 2011) with default settings except for the no_bowtie option. The raw reads from each library are available in the Sequence Read Archive database (SRA) with accession number: SRR7880397 to SRR7880408 and the assembled contigs are available in TSA database with accession number GGQE00000000 in BioProject: PRJNA492175 at the National Center for Biotechnology Information (NCBI, USA, http://www.ncbi.nlm.nih.gov/).

3.3.5 Functional annotation

Homology searches were carried out against UniProt release 2018_02 database, and Non-redundant protein (Nr) databases release 2017_09 using Blastx (version NCBI-blast-2.7.1+) software with a cut-off E-value of 1e-05 (Camacho et al., 2009). Gene ontologies were further analyzed using Blast2GO software (version 4.1.9) (Conesa and Götz, 2008) with default parameters to identify the best-represented biological processes detected in the reference transcriptome, based on the number of sequences included in each GO category. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to identify the genes involved in different metabolic pathways (Kanehisa and Goto, 2000).

3.3.6 Differential gene expression analysis

The reads from each library were aligned back to the reference transcriptome with Bowtie2 version 2.3.4.1 (Langmead and Salzberg, 2012). The estimation of transcripts abundance and normalization (fragments per Kilobase million, FPKM) was carried out with RSEM version 1.3.0 (Li and Dewey, 2011). The matrix built with the FPKM of all libraries was analyzed to obtain the differential expressed transcripts among treatments with DeSeq2 (False discovery rate, FDR < 0.05, fold change > 2) (Love et al., 2014). Four experimental conditions were defined to understand the relationship between the reproductive condition and thermal stress. Each treatment was compared (24POST, 30PRE and 30POST) against the control treatment (24PRE). The DE transcripts were arranged in clusters according to their expression pattern and represented in a heatmap in R software. The complete differential expression analysis was performed using the Perl and R scripts included in the Trinity package (instructions available in https://github.com/trinityrnaseq/trinityrnaseq/wiki/Post-Transcriptome-Assembly-Downstream-Analyses). Shared and exclusive transcripts among treatments were analyzed via Venn diagrams using VennDiagram package in R software.

3.3.7 Gene ontology (GO) enrichment analysis

The GO enrichment analysis for each transcript was performed to identify the possible biological processes in which these transcripts participate. An enrichment analysis (Fisher's exact test) was realized in Blast2GO to identify the best-represented categories in the biological process terms (p-value < 0.001).

3.3.8 Quantitative relative expression by real-time PCR.

Thirteen differentially expressed genes (DEGs) were selected for real-time PCR analysis in a CFX-96 system (Bio-Rad, USA) to validate the transcriptomics results. These genes were selected according to two criterions: A) their significant high expression and b) their importance in processes involved in the stress response and the reproductive processes.

We used the same RNA samples that were used for sequencing, and additionally, the samples obtained from males exposed to intermediate temperature (28 °C), PRE and POST mating conditions were included. cDNA was synthesized using ImProm-II™ Reverse Transcription System (PROMEGA) with 1.0 µg of total purified RNA in a total reaction volume of 20 µL (50 ng/µl) following the manufacturer´s protocol. The obtained cDNA´s were stored at -20 °C until use for PCR reactions. Gene-specific primers were designed using Primer3web software v.4.1.0 (Koressaar and Remm, 2007; Untergasser et al., 2012) based on RNA-Seq transcripts sequences. The primers sequences for each selected transcript are shown in Supplementary Table 10. The efficiency of the target and reference genes were calculated from a standard curve with an initial dilution factor of 1:5 and six subsequent serial dilutions with a factor of 1:2 of a cDNA pool including all experimental conditions. In our study, genes commonly used in the literature as housekeepings in other organisms (for example tubulins, elongation factors, actins, Glycerol-3-phosphate dehydrogenase) were significantly differentially expressed among the experimental conditions. For this reason, the housekeeping genes used in this study were chosen from the annotated transcript database of the assembled reference transcriptome. The selection was based on the lack of differential expression among treatments (FDR value = 1).

A total of 10 potential reference genes were evaluated for expression stability with the Genorm, Normfinder and Bestkeeper software, which results indicated that TUFM and TUBGCP were the most stable genes and were used as housekeeping genes for the relative expression analysis.

The qPCR reactions were carried out by triplicate with homemade Evagreen Mix 2x (Evagreen 20,000x in water, Biotium and AccuStart Taq DNA polymerase, Quanta, Beverly, MA). The reaction consisted in 5 µL of Evagreen Mix 2x, 0.2 µM of forward and reverse primers, 3 µL of cDNA template (dilution 1:5 or equivalent to 30 ng of total RNA) and 1.6 µL of sterile, free nuclease water, for a final volume of 10 µL. The thermal cycling conditions were 94 °C for 3 min, followed by 40 cycles at 94 °C 30 s, annealing temperature for 30 s (Supplementary Table 10) and 72 °C for 30 s. A melt curve analysis was included at the end (95 °C for 10 s, 65 °C to 95 °C for 5 s, with increments of 0.5 °C) to corroborate PCR products specificity. The amplicons length were confirmed in agarose gel electrophoresis at 1.5%. Relative expression (RE) of target genes was estimated using the $\Delta\Delta$ Cq method, as proposed by Hellemans et al., (2007). For statistical analysis, all RE values were transformed to the logarithm (log10), and a two-way ANOVA model was used to establish the effects of temperature and mating condition, with a statistical significance of $P<0.05$. A *post hoc* analysis of means was done using Fisher's LSD test. All statistical analyses were performed using STATISTICA 6.1 (StatSoft, Tulsa, OK, USA).

3.4 Results

3.4.1 Transcriptome sequencing, trimming and assembly

The sequencing of all the testis libraries generated 53,214,611 paired-end raw reads with a length of 75 bp. After discarding Illumina adaptors and reads with low quality, a total of 48,101,426 reads with a Phred score over 30, were de novo assembled to generate the reference transcriptome using Trinity. Table 7 summarizes the number of sequenced reads and the trimming statistics per sample. The *de novo* assembled testis transcriptome consisted in 53,848,027 bases. The contigs length ranged from 201 nt to 12,758 nt with an average length of 631 nt, N50 = 889 nt (based on all transcript contigs) and GC content of 38%. A total of 85,249 transcripts (including all isoforms) and 77,661 genes were reconstructed (Table 8). The *de novo* testis transcriptome of *O. maya* was deposited at the NCBI. From the reference testis transcriptome, a total of 915 transcripts were exclusively expressed in the control treatment (24PRE), 923 in 24POST, 1,492 in 30PRE and 2,002 in 30POST (Figure 7A).

Table 7. RNA-Seq reads obtained on Illumina MiSeq system.

Sequencing statistics	Number of raw reads before trimming	Number of raw reads after trimming	Raw reads after trimming (%)
24PRE-1	4,261,482	3,715,125	87.18
24PRE-2	3,801,367	3,514,073	92.44
24PRE-3	3,783,317	3,509,953	92.77
24POST-1	4,144,156	3,634,299	87.70
24POST-2	4,529,867	3,997,421	88.25
24POST-3	3,908,012	3,596,089	92.02
30PRE-1	5,792,353	5,164,041	89.15
30PRE-2	4,160,642	3,705,038	89.05
30PRE-3	4,933,884	4,580,123	92.83
30POST-1	4,648,617	4,135,854	88.97
30POST-2	4,822,915	4,452,090	92.31
30POST-3	4,427,999	4,097,320	92.53
Total	**53,214,611**	**48,101,426**	**90.39**

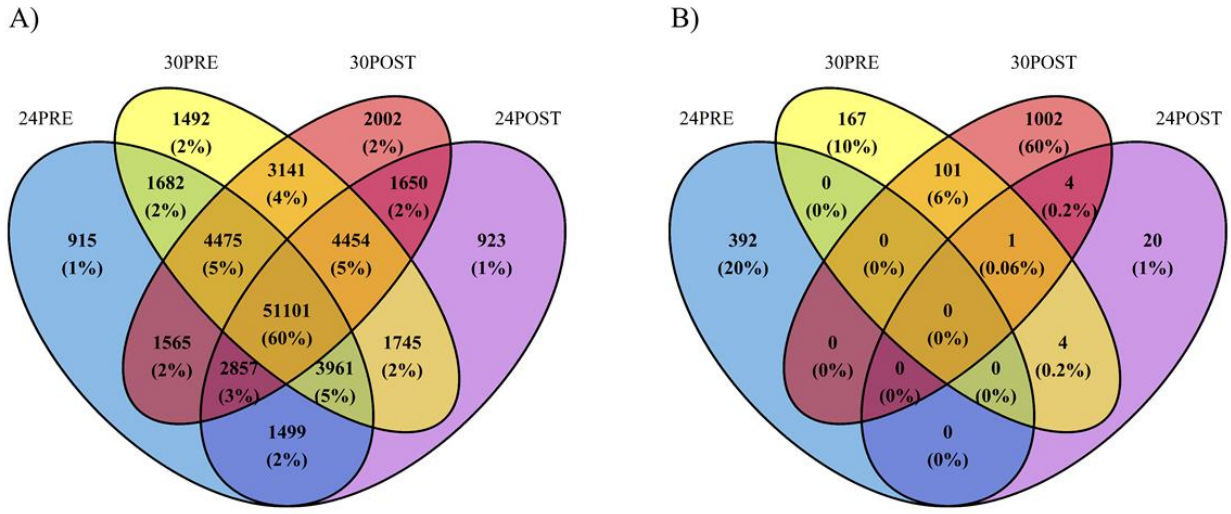

Figure 7. (A) Venn diagram of the number of transcripts expressed in the reference testis transcriptome of *Octopus maya* in each treatment. (B) Venn diagram of DEG's with significantly higher expression in each treatment. Treatments: 24PRE – control treatment exposed to 24 °C; 24POST – mated and exposed to 24 °C; 30PRE – exposed to 30 °C; 30POST – mated and exposed to 30 °C.

Table 8. *De novo* Assembly and annotation statistics.

Trinity Assembly statistics	**All contig transcripts**
Total assembled bases	53,848,027
Total number of transcripts	85,249
Total number of genes	77,661
GC Content (%)	38
Contig N10	2,949
Contig N20	2,118
Contig N30	1,604
Contig N40	1,204
Contig N50 (based on all transcript contigs)	889
Contig N50 (based on longest contig isoform)	783
Median contig length (nt)	381
Average transcript length (nt)	631
Total transcripts with ORF	14,331
Max length (bp)	12,758
Min length (bp)	201
Number of transcripts over 1 Kb	14,331
Annotation statistics	
Annotated transcripts by UniProt	13,154 (15.4%)
Contigs with Cellular Component terms	10,856 (82.5%)
Contigs with Biological Process terms	10,663 (81.1%)
Contigs with Molecular Function terms	10,535 (80.1%)
Annotated transcripts by Nr	11,151 (13.1%)
Annotated transcripts by KEGG	5,461 (6.4%)

3.4.2 Functional annotation of *Octopus maya* testis transcriptome

The transcripts were annotated by comparing with Nr, UniProt and KEGG databases. In total, 16,804 (19.7%) and 31,555 (37.0%) transcripts had at least one significant homolog against proteins of the UniProt and Nr databases, respectively (e-value cut-off: 1e-5). Most of the sequences with homology against those databases had an e-value among 1e-05 to 1e-45. The 51% of the homologous sequences obtained from UniProt presented a similarity distribution among 60% to 80%, while the 76% of the homologous found in the Nr had a similarity distribution among 80-100%. Figures 8A and 8B show the e-value and the similarity distribution for the blastx hits against UniProt and Nr databases. From the blast hits obtained with the

UniProt database, the higher number of matches corresponded with sequences of *Homo sapiens* (37%) followed by sequences of *Mus musculus* (24%), *Rattus norvegicus* (7%), and *Bos Taurus* (7%) (Figure 8D). In the case of the hits matched with the Nr database, the highest number of matches corresponded to sequences of *Octopus bimaculoides* (96%) (Figure 8C). This high identity percentage suggests that the *O. maya* gene fragments were correctly assembled and annotated.

We applied the Blast2GO algorithm to classify the transcripts in functional categories: biological process, molecular function and cellular component. The results showed that only 13,154 transcripts (15.4%, UniProt database) and 11,151 (13.1%, Nr database) of 85,249 transcripts had at least one GO term assignation and could be annotated (Table 8).

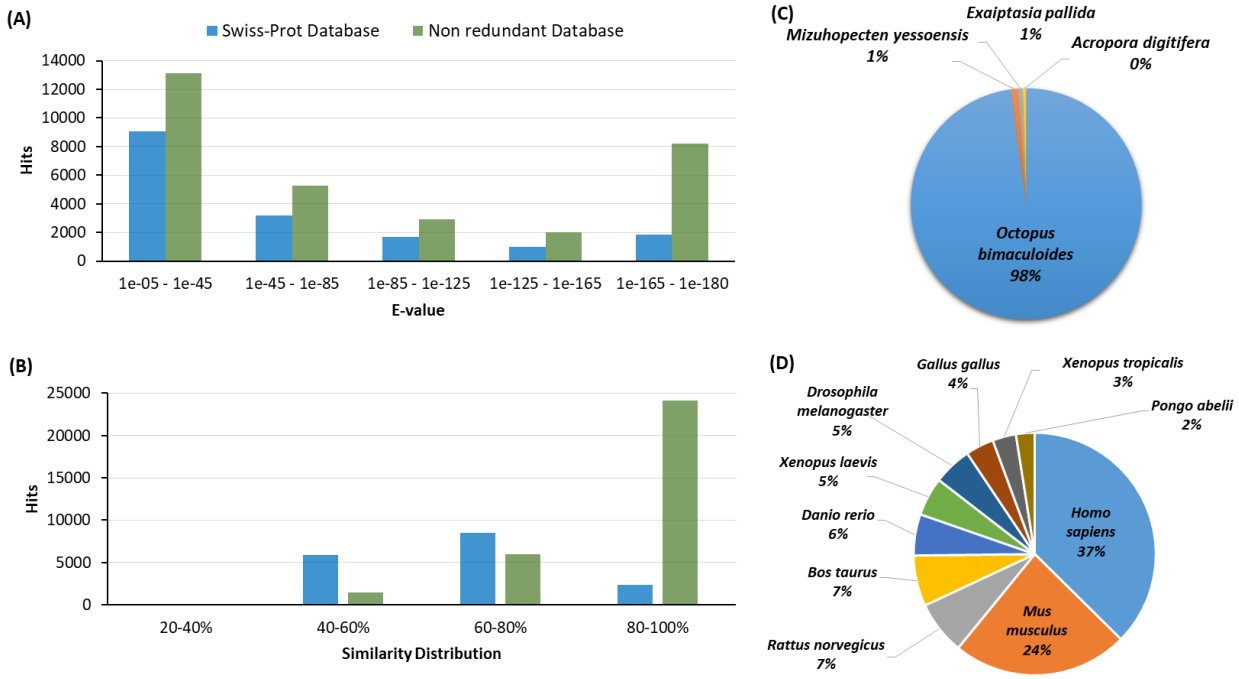

Figure 8. (A) E-value distribution of the Blastx hits against the UniProt and Non-redundant (Nr) database for each transcript. **(B)** Similarity distribution of the Blastx hits against the UniProt and Nr database. **(C)** Species distribution of the top blast hits of the transcripts in the Nr database in the testis transcriptomic analysis of *Octopus maya* males. **(D)** Species distribution of the top blast hits of the transcripts in the UniProt database in the testis transcriptomic analysis of *Octopus maya* males.

The Gene Ontology (GO) assignments carried out at level 3 revealed that most of the sequences were categorized in cellular components (10,856; 82.5%), followed by biological processes (10,663; 81.1%) and molecular functions (10,535; 80.1%). The biological processes identified were cellular metabolic process (6,764 transcripts; GO:0044237), response to stress (1,184 transcripts; GO:0006950), cell cycle (1,279 transcripts; GO:0007049), microtubule-based process (530 transcripts; GO:0007017), response to abiotic stimulus (445 transcripts; GO:0009628), cell motility (444 transcripts; GO:0048870), chromosome segregation (174 transcripts; GO:0007059), immune response (316 transcripts; GO:0006955), sperm part (64 transcripts; GO:0097223) and meiotic cell cycle (90 transcripts; GO:0051321). The main cellular components identified were intracellular (9,594 transcripts; GO:0005622), membrane-bounded organelle (6,855 transcripts; GO:0043227), endomembrane system (2,026 transcripts; GO:0012505) and protein complex (1,640 transcripts; GO:0043234). The main molecular functions identified were protein binding (5,139 transcripts; GO:0005515), hydrolase and transferase activity (2,448 and 2,257 transcripts, respectively; GO:0016787 and GO:0016740; Figure 9).

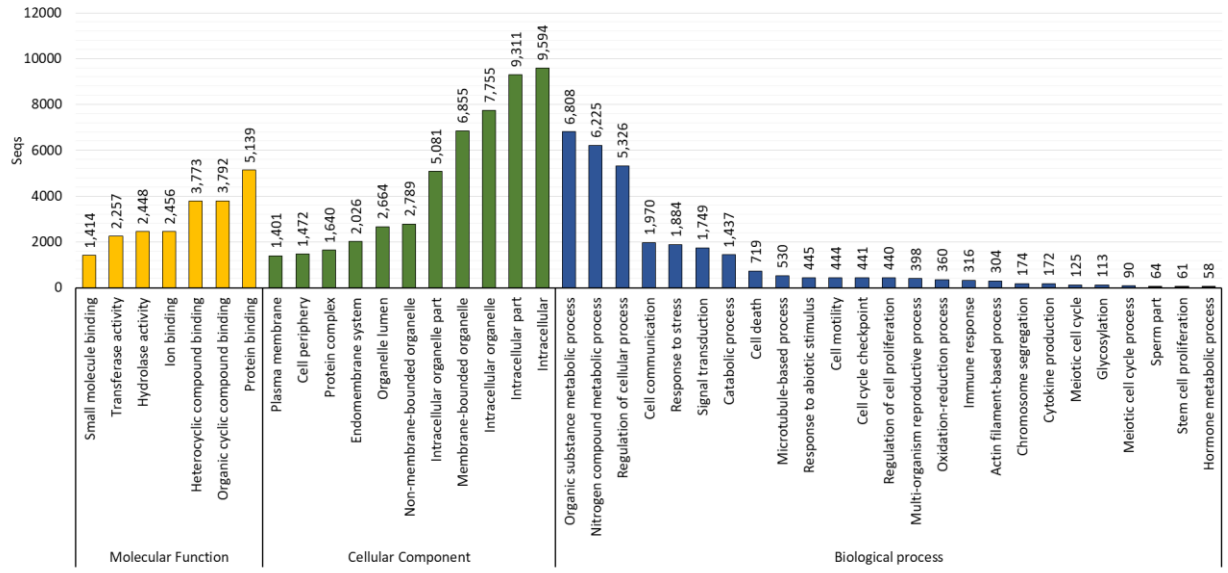

Figure 9. Gene ontology (GO) distribution by category at level 3 in the reference testis transcriptome of *Octopus maya* males.

A total of 3,317 transcripts (25.2%) matched with homologous proteins in the KEGG database associated with 130 distinct KEGG pathways. Among the top five categories, nucleotide, cofactors and vitamins metabolism are the largest represented classes, and the top-hits pathways in these categories were purine and thiamine metabolism with 705 transcripts.

3.4.3 Differentially expressed genes (DEGs)

The heatmap of DEGs detected in each library is shown in Figure 10. The expression patterns revealed that 24PRE and 24POST had a similar expression pattern, which evidences that copula did not affect gene expression under optimal thermal condition. In 30PRE treatment, it was observed some genes (273) with significantly higher expression in comparison to the control treatment (24PRE). At this point, we found that thermal increment modifies the expression patterns in the testis. In the case of 30POST, we observed the major number of genes with higher expression in comparison to the control treatment. The 30POST condition showed an expression profile opposite to the control. This pattern evidences that the combined effect of high temperatures and the reproductive activity has a significant effect over the molecular mechanisms that regulate gene expression in the testis of *O. maya* males.

The differential expression analysis showed 1,881 significantly differentially expressed transcripts using 24PRE as the control treatment. A total of 1,410 transcripts were significantly more abundant (29 transcripts in 24POST, 167 transcripts in 30PRE and 1,002 transcripts in 30POST; $P < 0.05$, $FC > 2$) in all treatments vs the control. Figure 7B shows the Venn diagram of the transcripts with significantly higher expression in the treatments that had homologs with the UniProt database. A total of 471 transcripts were significantly more abundant in the control vs all the treatments (16 transcripts vs 24POST, 160 transcripts vs 30PRE and 295 transcripts vs 30POST; $FDR < 0.05$, $FC > 2$). We also found differentially expressed transcripts that did not match the protein databases, a total of 13 in 24POST, 176 in 30PRE and 977 in 30POST. Even, some of these transcripts have higher expression than those putative transcripts with homologs in the peptide databases.

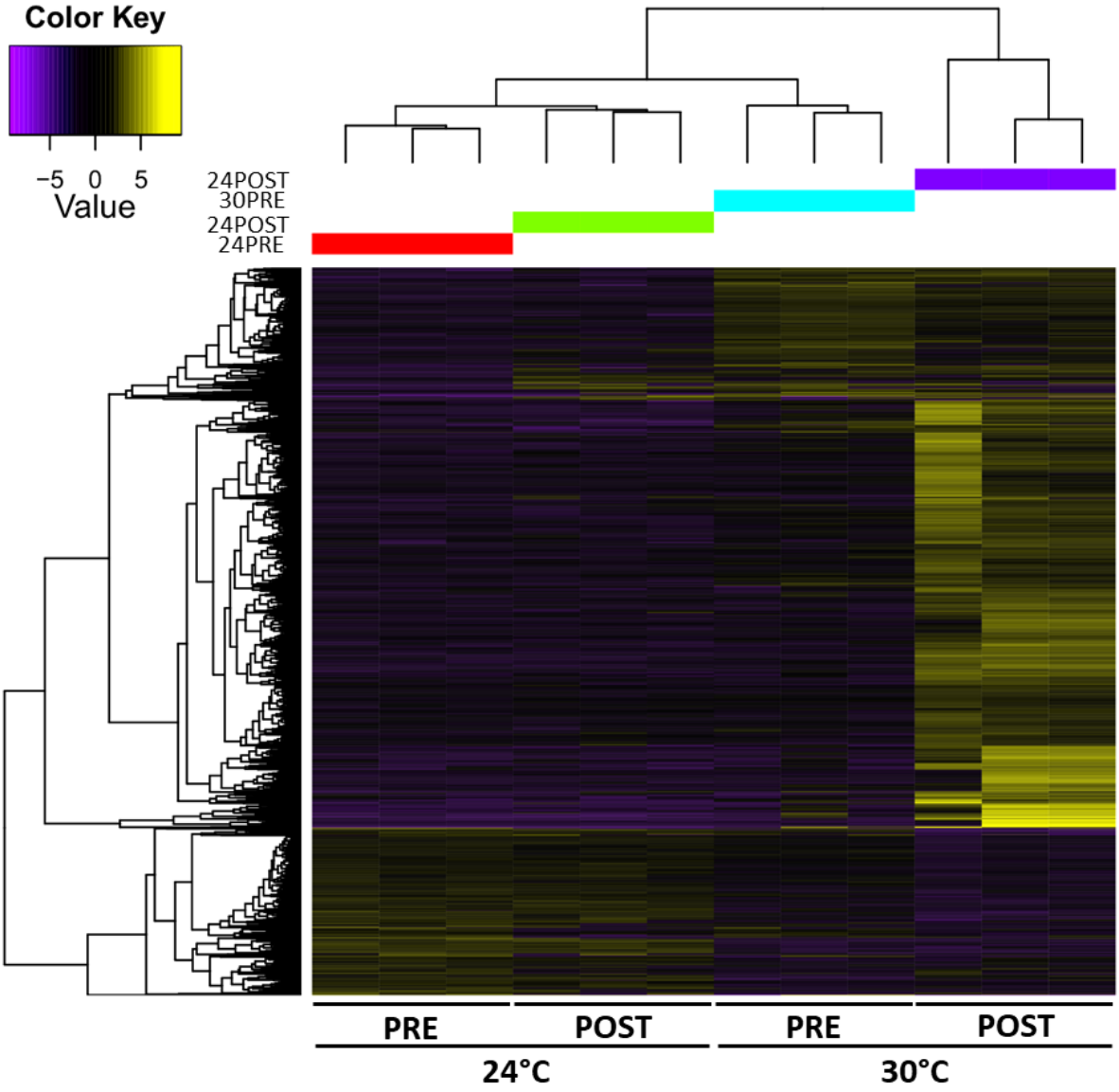

Figure 10. Heatmap of the abundance of differentially expressed genes (rows, FDR < 0.05, Fold change > 2) in the *Octopus maya* testis transcriptome in each treatment (columns). The dendrogram shows that temperature modulated the expression patterns. Treatments: 24PRE – control treatment exposed to 24 °C; 24POST – mated and exposed to 24 °C; 30PRE – exposed to 30 °C; 30POST – mated and exposed to 30 °C.

3.4.4 GO Enrichment analysis

Biological processes with significant enrichment ($P < 0.05$) were detected in each thermal and reproductive condition by using the transcripts with higher expression in each treatment. In 24POST, 16 of the 29 upregulated DEGs significantly enriched 146 biological process categories (Figure 11), while nine of the 16

downregulated DEGs significantly enriched a unique biological process category. In 30PRE, 96 of the 273 upregulated DEGs significantly enriched 396 biological processes (Figure 12), while 76 of the 160 downregulated DEGs significantly enriched21 biological processes. In 30POST, 531 of the 1,108 upregulated DEGs enriched 390 biological processes significantly (Figure 13), while 129 of the 295 downregulated DEGs significantly enriched 67 biological process categories. The genes that best represented the enriched biological processes involved in stress response and reproductive process are shown in Supplementary Table 11 and 12.

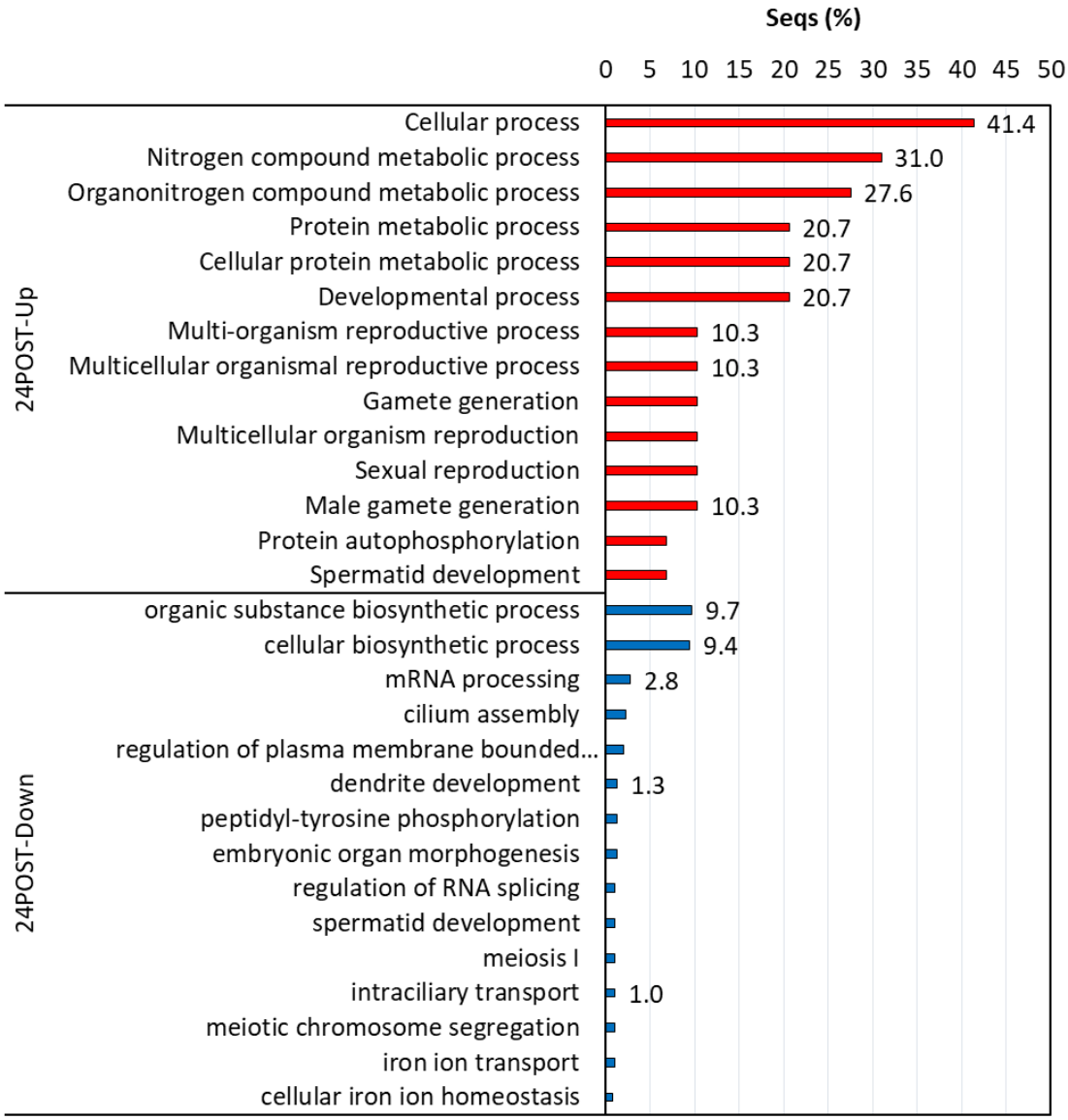

Figure 11. Enriched GO terms of biological processes (Fisher exact test, FDR < 0.05) in 24POST treatment vs the control treatment (24PRE). Up (red) and down-regulated (blue) transcripts are shown.

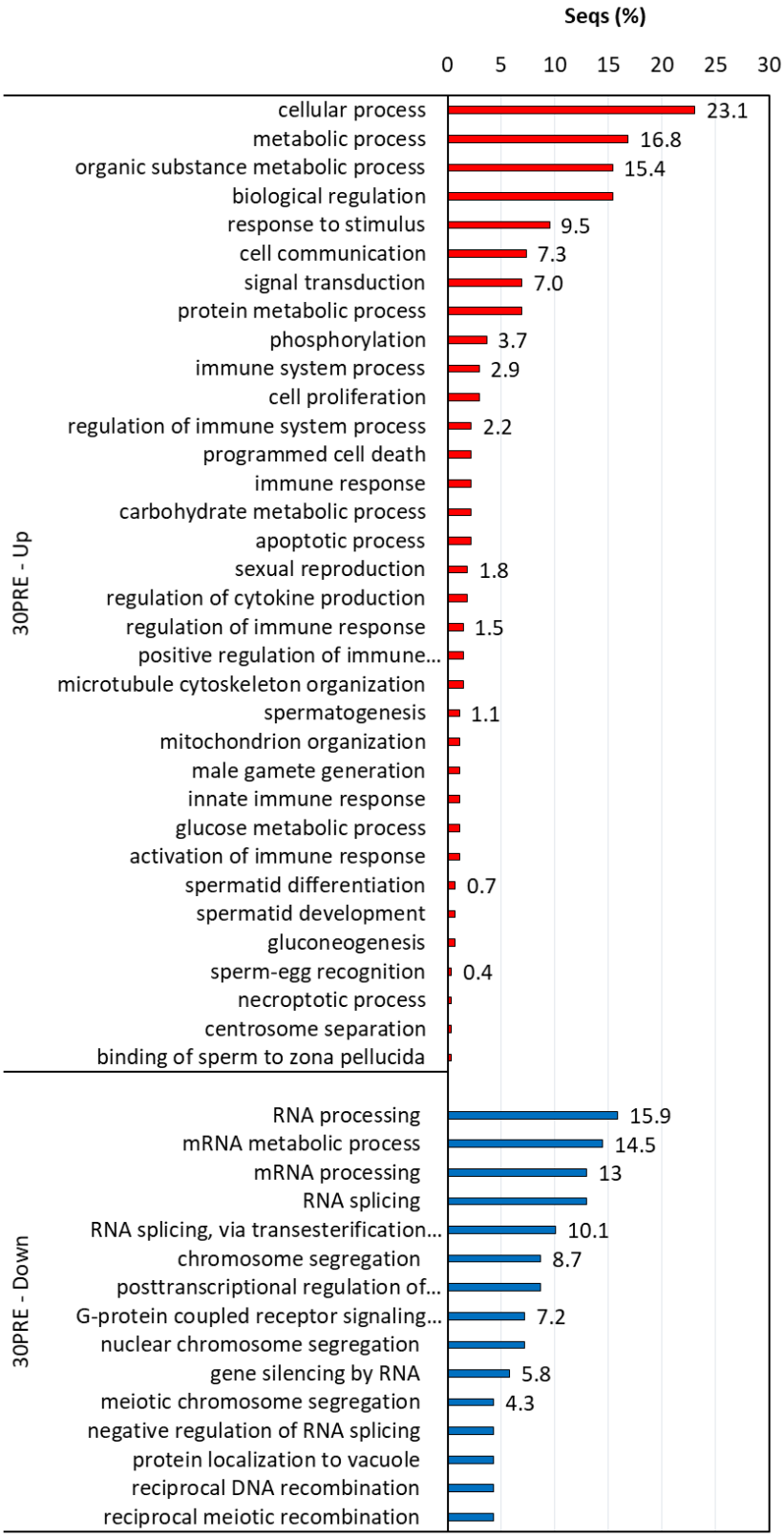

Figure 12. Enriched GO terms of biological processes (Fisher exact test, FDR < 0.05) in 30PRE treatment vs the control treatment (24PRE). Up (red) and down-regulated (blue) transcripts are shown.

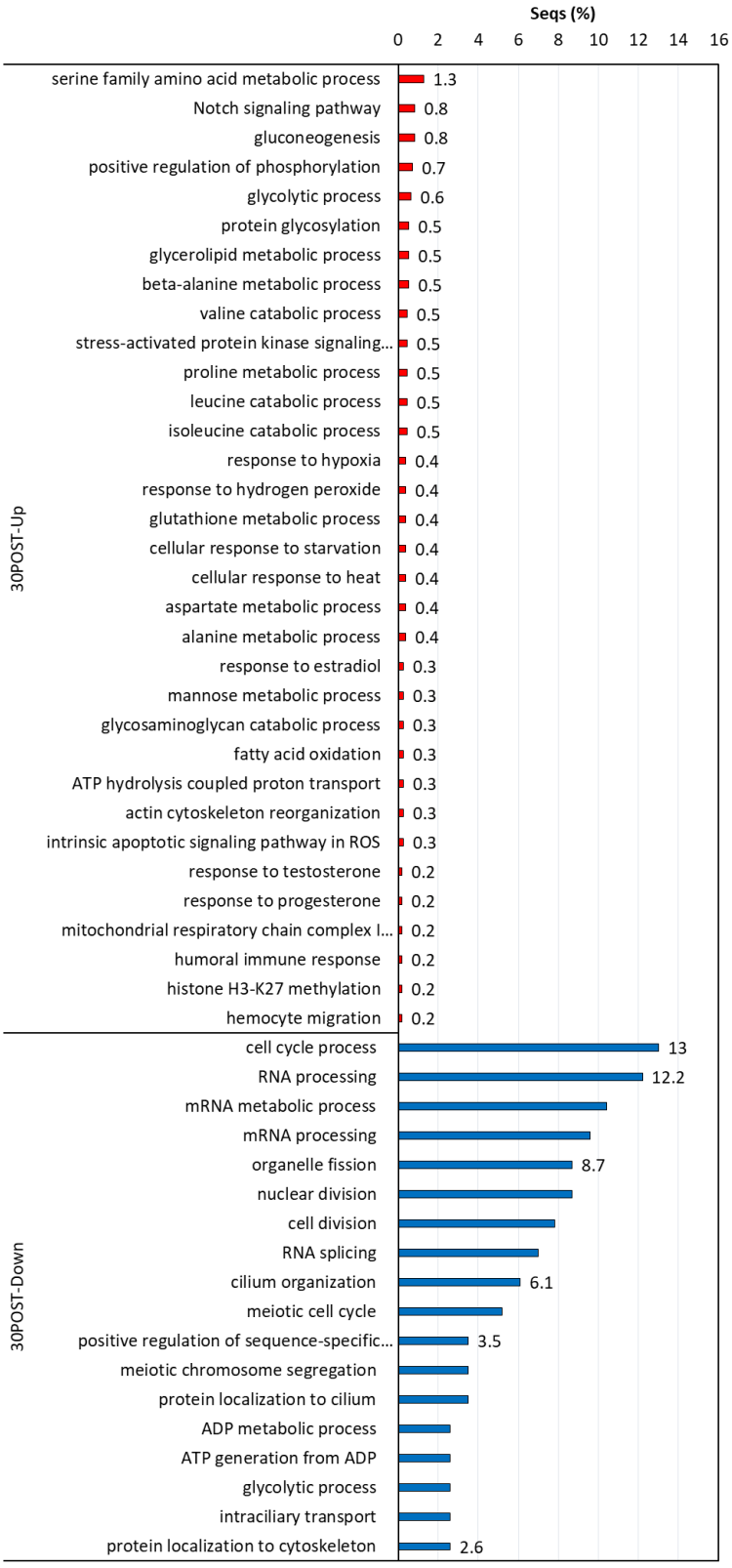

Figure 13. Enriched GO terms of biological processes (Fisher exact test, FDR < 0.05) in 30POST treatment vs the control treatment (24PRE). Up (red) and down-regulated (blue) transcripts are shown.

3.4.5 Stress response

3.4.5.1 Transcripts involved in response to thermal (TS) and oxidative stress (OS)

All the differentially expressed transcripts involved in the thermal stress response were highly expressed at 30 °C (Figure 14A). In the 30PRE condition, the gene SH3RF1 (TS) showed higher expression in comparison to 30POST. The genes such as CRIP1, ITGA9, SLC8A3, FLNA, DDXN1 and PDCD6 (ST) were conspicuous in 30POST condition. The genes ABR, SETMAR, UBC6, BABAM1 and C3 (TS) were induced in both conditions.

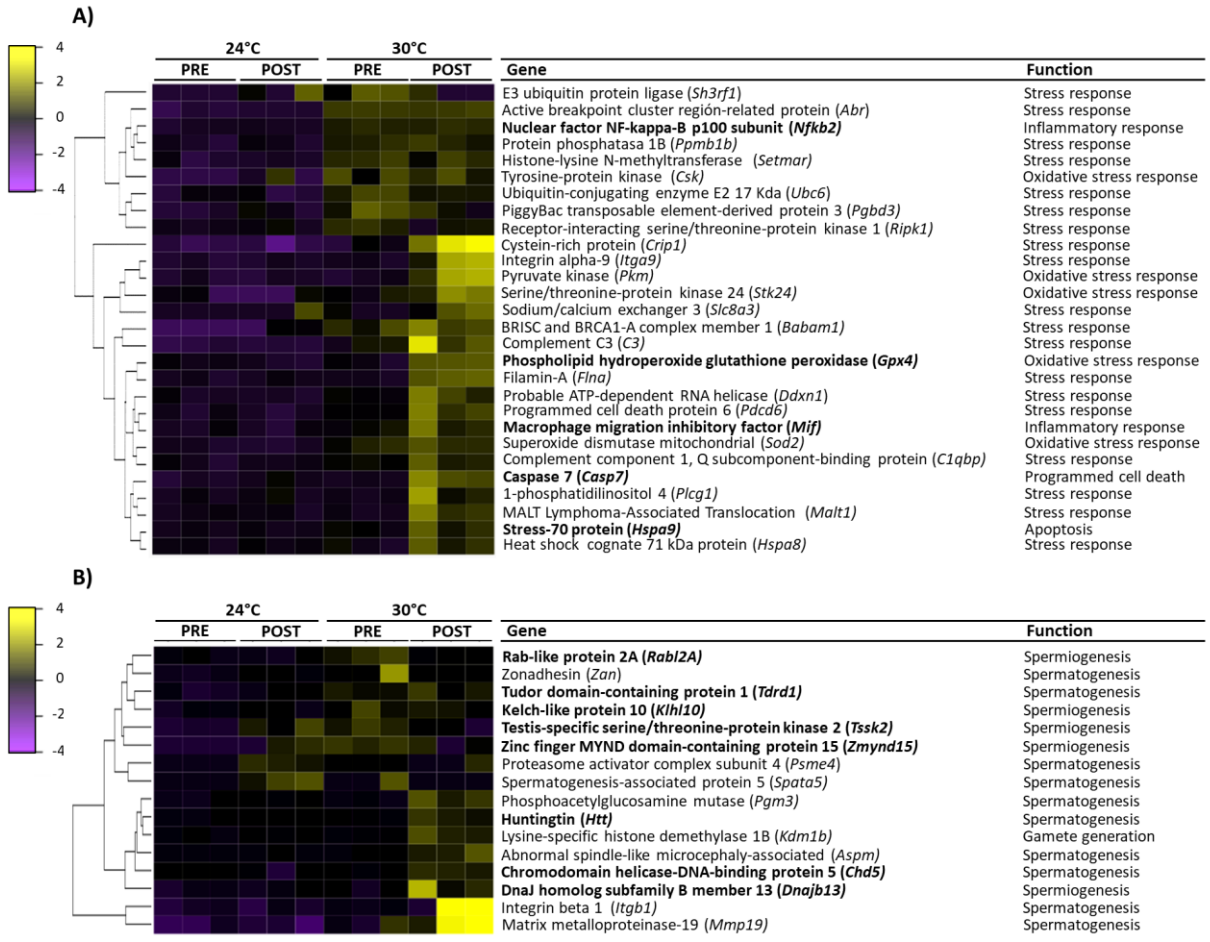

Figure 14. Heatmap is representing the expression values of differentially expressed genes (FDR < 0.05, Fold change > 2) between 24PRE, 24POST, 30PRE and 30POST treatments and their main function. (A) Genes involved in the stress response. **(B)** Genes involved in the reproductive process. Sample names are represented in columns, and significant genes are represented in rows. Genes are clustered together based on expression similarity. Low to high expression is represented by a change of colour from purple to yellow, respectively.

The genes involved in the oxidative stress response as CSK and SOD2 were highly abundant in both treatments (30PRE and 30POST), while the genes PKM, STK24, GPX4 and SOD2 were highly expressed specifically in 30POST treatment (Figure 14A).

3.4.5.2 Transcripts involved in cytokine production, inflammatory process, apoptosis, and necroptosis.

The genes that best-represented these categories were exclusively induced at 30 °C and specific changes were observed between PRE and POST condition (Figure 14A). The genes involved in the cytokine production as PPMB1, CSK and PGBD3 showed higher expression in both 30PRE and 30POST conditions, meanwhile C3, and BCL only in 30POST condition. The genes up-regulated in inflammatory processes as MIF, MAPKAPK2 and CHIA presented higher expression in 30POST and NFKB2 only in 30PRE condition. The genes HSPA9, TRAF2 and TNIP2, showed higher expression 30POST condition, and SH3GLB1 and TFDP1 in 30PRE. The gene RIPK1 (necroptotic process) presented higher expression in 30PRE condition. The gene CASP7 was highly abundant in the condition 30POST.

3.4.6 Reproductive process

3.4.6.1 Transcripts involved in spermatogenesis, spermiogenesis and gamete generation processes

The genes that best-represented these biological processes were exclusively induced at 30 °C, and specific changes were observed between PRE and POST condition (Figure 14B). The genes involved in the spermatogenesis process as ZAN and TDRD1 showed higher expression at 30 °C under PRE and POST conditions meanwhile PSME4 showed higher expression in 24POST and 30PRE treatments. SPATA5 showed unique higher expression in 24 °C after copula. The up-regulated genes PGM3, HTT, ASPM, CHD5, ITGB1 and MMP19, showed the highest expression in 30POST treatment. The genes involved in spermiogenesis as RABL2, KLHL10 and TSSK2 showed higher expression in 30 °C PRE and POST conditions meanwhile ZMYND15 showed higher expression in 24POST and 30PRE treatments. The up-regulated

DNAJB13 gene showed the highest expression in 30POST. The KDM1B gene (gamete generation) process was highly expressed in 30POST treatment.

3.4.7 qPCR validation

The Pearson correlation coefficient was measured in the RNA-seq and qPCR data. The correlation coefficient for *Gpx* (r=0.91)*, Hspa9 (*r=0.90)*, Casp7* (r=0.99)*, Nfkb2*(r=0.95) and *Mif* (r=0.94) revealed that relative expression measured by qPCR is consistent with the RNAseq data (Supplementary Figure 20). The expression pattern of the up-regulated genes involved in the reproductive process was confirmed by qRT-PCR (Supplementary Figure 21). The results revealed high correlation values for *Klhl10* (r=0.99)*, Htt* (r=0.99), *Tdrd1*(r=0.80) and *Rabl2a* (r=0.83).

Different transcripts presented in oxidative stress response (GPX4), inflammatory processes (MIF, NFKB2) apoptosis (CASP7, HSPA9) were selected for relative expression analysis by qPCR to validate the differential expression results described above. Consistent with the RNA-seq data, transcripts as GPX4, CASP7, HSPA9 and MIF showed a higher expression in 30POST condition. Meanwhile, NFKB2 presented the highest expression at 30 °C compared to 24 °C PRE condition. Additionally, when the intermediate temperature was included in the expression analysis, ANOVA results for stress response-related genes (Figure 15) indicated that temperature has a significant effect on the expression levels of GPX4 (P=0.0029), CASP7 (P=0.0005), HSPA9 (P=0.0026) and NFKB2 (P=0.0006). On the other hand, differences between mating condition were detected only for GPX4 (P=0.0003) and CASP7 (P=0.0085), with the interaction between temperature and condition being also significant for both transcripts (P<0.05). This result was mainly caused by the expression in PRE 28 °C, which was significantly higher than that at 24 °C and 30 °C in the same reproductive condition. Meanwhile, in POST condition, the expression was significantly higher at 30 °C respectively to that observed at 24 °C and 28 °C. No significant differences between mating conditions were observed for HSPA9 (P=0.96) and NFKB2 (P=0.99), nor for the interaction between factors (P>0.05). Finally, the relative expression of MIF did not show significant differences between temperature (P=0.067), condition (P=0.44) or the interaction between them (0.537), although its expression appeared to be increased with temperature in both conditions.

The expression pattern of the eight highly expressed genes involved in the reproductive process (KLHL10, TSSK2, DNAJB13, RABL2A, CHD5, ZMYND15, TDRD1 and HTT) were confirmed by qRT-PCR (Figure 16). For

HTT, the highest expression was observed in 30POST, whereas for TDRD1and KLHL10 both PRE and POST condition showed the highest expression. In the case of RABL2A, 30PRE condition showed higher expression than 24PRE. All these results were consistent with differential expression analysis of the transcriptome.

In order to determine the role of temperature and mating condition in *O. maya* in the expression pattern of the selected genes, the 28 °C temperature was included in the analysis. This temperature allowed to find that there was a significant effect of temperature in the expression of KLHL10 ($P<0.01$), RABL2A ($P=0.009$), CHD5 ($P=0.025$), TDRD1 ($P<0.01$), ZMYND15 ($P=0.036$) and HTT ($P=0.002$). However, different patterns were observed regarding the reproductive condition and the interaction between the factors. For KLHL10 and RABL2, no effect of mating condition ($P>0.05$) or the interaction between factors ($P>0.05$) was observed, indicating that expression between temperatures has similar patterns in PRE and POST mating, being significantly higher in average at 30 °C for both transcripts. On the other hand, for CHD5 no significant differences between PRE and POST condition were observed ($P=0.136$), but the significance of the interaction between temperature and mating conditions ($P=0.0352$) was caused by the significantly lower expression at 28 °C in POST condition. For TDRD1 the expression in PRE condition was significantly higher on average respect to POST ($P=0.001$), but no significant interaction was observed ($P=0.944$) indicating that for PRE and POST condition the expression of TDRD1 has a similar pattern between temperatures with the lowest expression observed at 24 °C.

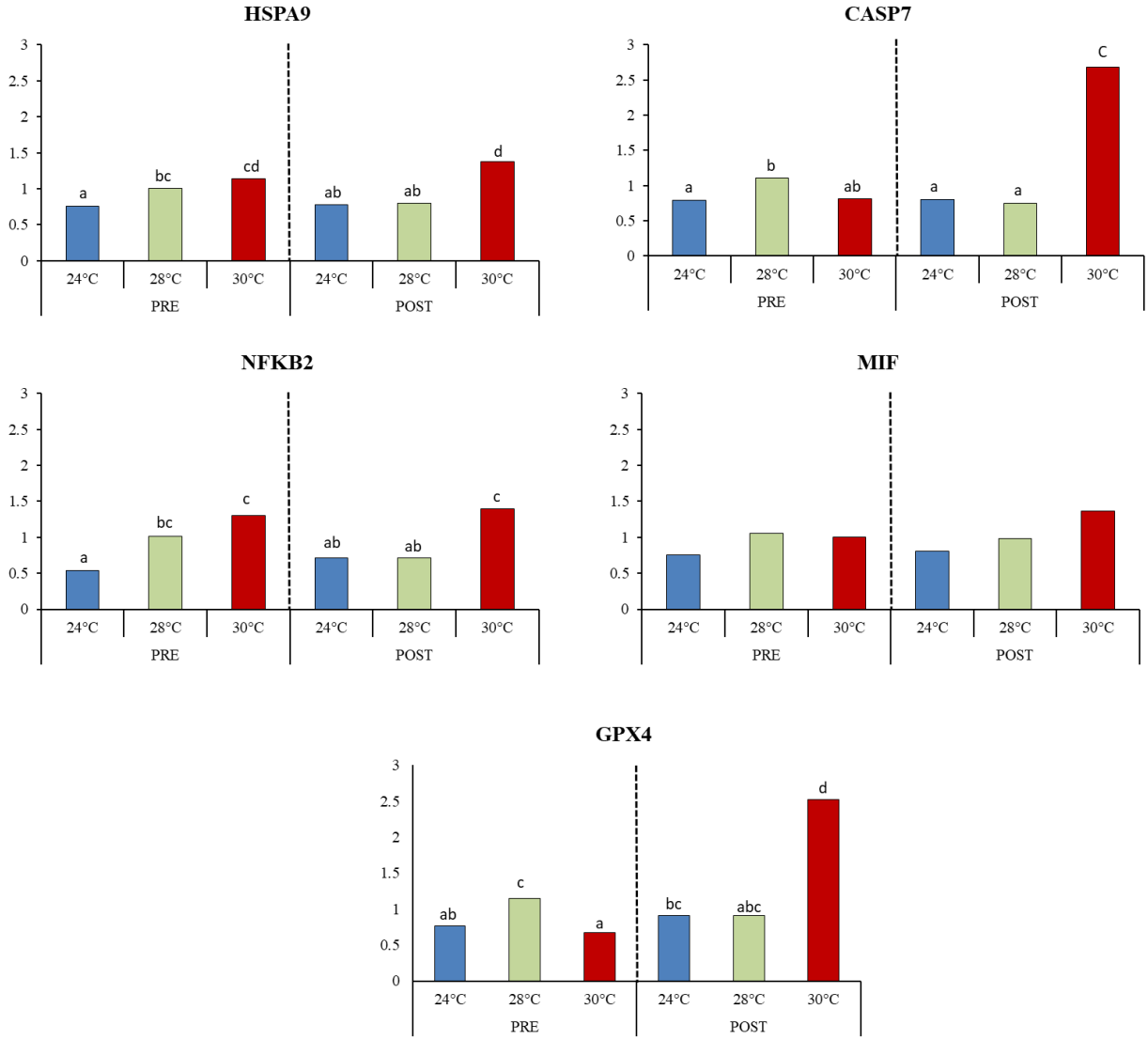

Figure 15. Relative expression of transcripts associated with thermal stress response. Mean values (back-transformed from logarithms) are shown in bars and letters are used to denote differences between means ($P < 0.05$).

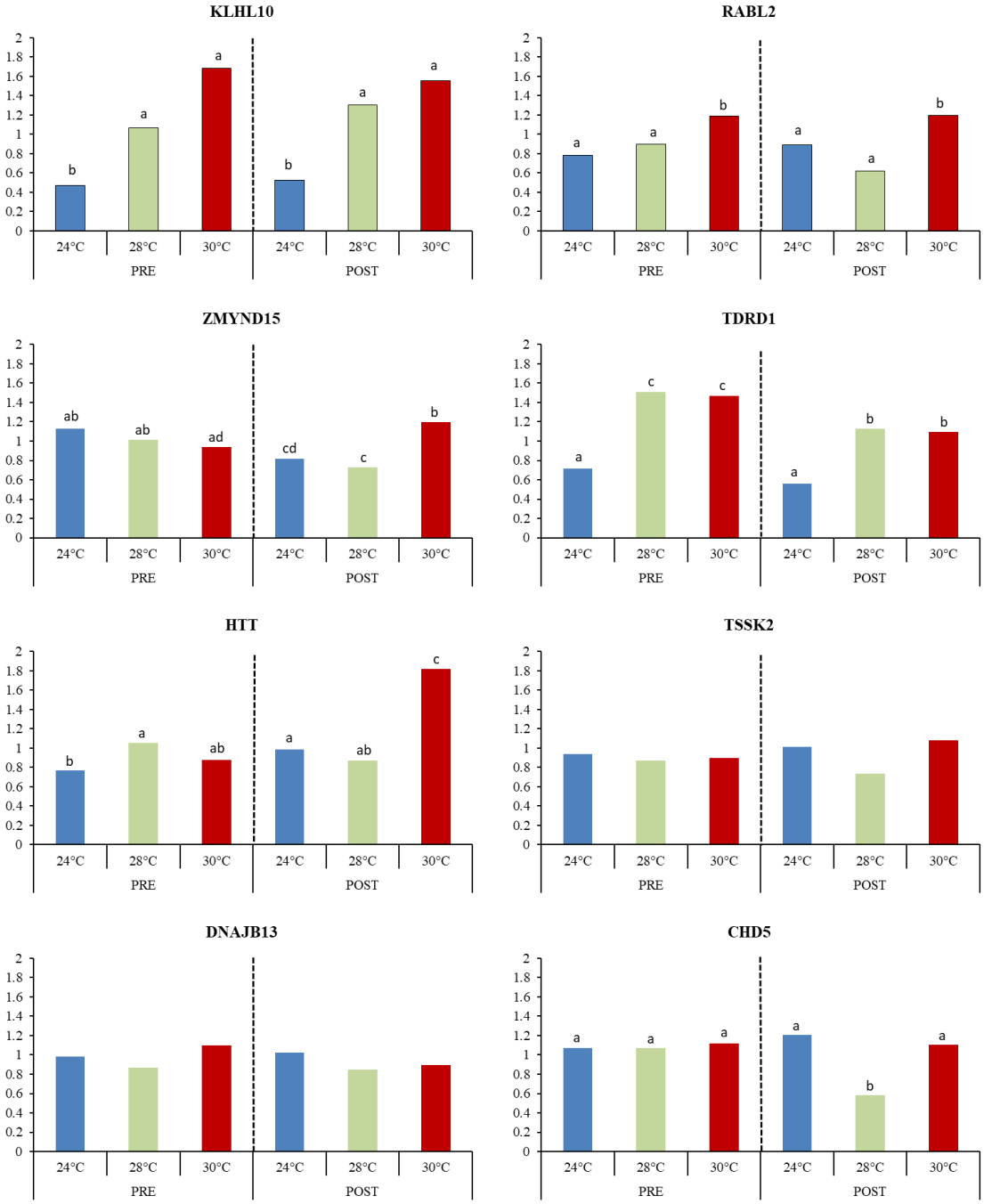

Figure 16. Relative expression of transcripts associated with the male reproductive process. Mean values (back-transformed from logarithms) are shown in bars and letters are used to denote differences between means ($P < 0.05$).

3.5 Discussion

Octopus maya as an ectotherm organism is strongly influenced by temperature (Noyola et al., 2015). Temperature plays an important role in different life aspects as embryo development, growth patterns, morphology, physiology and reproduction. As an endemic species of the Yucatan peninsula (YP), is influenced by the thermal characteristics of the platform, where temperatures can vary since 21 °C to 30 °C along the year (Noyola et al., 2013a, 2013b). In this study, male octopuses were exposed at three temperatures 24 °C (Optimal), 28 °C (intermediate) and 30 °C (Stress) during 30 days, and then, a group of males for each experimental temperature were mated with females acclimated at 24 °C. An RNA-Seq analysis of the testis transcriptome at contrasting temperatures (24 °C and 30 °C) was realized to evaluate the transcriptomic responses to chronic thermal stress and the mechanisms involved in the regulation of reproductive processes. In recent years, the high-throughput sequencing techniques have allowed obtaining genetic and genomic information of both model and non-model organisms, the latter in which there are no (or very limited) genomic resources (Ekblom and Galindo, 2011). This technique allows evaluating the expression profiles of a large number of genes, robustly. In our RNA-seq analysis, we used pooled samples to minimize the effects of biological variation in treatments. We obtained de novo transcriptome of *O. maya* testis constituted 77,661 genes reconstructed. The main species that matched our blast hits against UniProt and Nr Databases were *Homo sapiens* and *O. bimaculoides*, respectively. This match is completely attributable to the big number of known proteins of model organisms like *Homo sapiens* in the UniProt database and in the case of *O. bimaculoides,* the recent release of its genome (Albertin et al., 2015)), and the close phylogenetic relationship between *O. maya* and *O. bimaculoides* (Juárez et al., 2012). The GO functional annotation showed 13,154 (15.4%) and 11,151 (13.1%) transcripts with homologies in the UniProt and Nr databases, with similar proportion to that found in other cephalopods as *Octopus vulgaris* (Castellanos-Martínez et al., 2014; Zhang et al., 2012), *Euprymna tasmanica* (Salazar et al., 2015) and *Sepia officinalis* (Cornet et al., 2014). The fact that the 84.6% and 86.9% (UniProt and Nr databases) of the transcripts did not match any known proteins suggests that there may be a high number of potentially novel genes in *O. maya* that remain to be properly characterized. In this study, 1,166 transcripts without homology were differentially expressed among treatments with high expression values; more studies have to be done to characterize these novel genes. This lack of molecular data emphasizes the importance of cephalopods studies to elucidate the molecular mechanisms involved in their physiology, development, growth and reproduction.

In general, in the reference testis transcriptome, we identified putative genes involved in the biological process as metabolism, stress response, cell cycle, microtubule-based process, sperm part and

chromosome segregation. These results indicated that some important traits inherent to the organisms as metabolic activity, cellular response and cellular processes occurred in *O. maya* testis during chronic thermal stress and mating activity.

3.5.1 Genes related to thermal stress response in *O. maya* testis transcriptome.

One of the goals in the present study was to assess the presence of genes involved in stress response, apoptosis and inflammatory processes in the *O. maya* testis transcriptome to confirm that thermal stress affects the molecular mechanisms that regulate the reproductive performance and success of this species. There are different heat stress response mechanisms as DNA reparation, heat shock response, antioxidant defense, cell cycle checkpoints and apoptosis (Pérez-Crespo et al., 2008). The apoptosis process in the testis is characterized by the apparition of acidophilic bodies. These acidophilic bodies under normal conditions of spermatogenesis, maintain the equilibrium between cellular proliferation and apoptotic degeneration (William et al., 1997). Apoptosis process has been well understood in humans with infertility issues, where an increment in the process such as maturity arrest and hypospermatogenesis has been observed (William et al., 1997). In this study, we identified necroptotic processes in male octopus exposed to 30 °C; this is coincident with the severe testicular damage observed at 30 °C, an increment four times higher than that of those exposed to 24 °C, and dilation of germ cells strata (López-Galindo et al., 2019). The necrosis is a process of programmed cell death caused by external factors which trigger an immune response characterized by the inflammatory process (Shaha et al., 2010). In this sense, the gene ontology analysis of the *O. maya* testis transcriptome revealed the presence of genes involved in regulation of cytokine production (PPMB1, C3, CSK, PGBD3, BCL3) which are cell signalling proteins that regulate the inflammation and infection in the body (Castellanos-Martínez et al., 2014); inflammatory process which is important for a rapid and efficient elimination of damaged tissue (NFKB2, MIF, MAPKAPK2, CHIA2) (Ottaviani et al., 2010), apoptosis which is a process characterized by dying cells, cytoplasmatic shrinkage, active membrane blebbing, chromatin condensation, and typically, fragmentation into membrane-enclosed vesicles or apoptotic bodies (HSPA9, SH3GLB1, TFDP1, TRAF2, TNIP2); and necroptotic process where necrosis is characterized by cytoplasmic and organelle swelling, and plasma membrane rupture (RIPK1) (Peterson et al., 2015). The necroptosis has been recently investigated. This form of necrosis is dependent of the kinases RIP1 and RIP3, and a pseudokinase MLKL (Peterson et al., 2015). The differential expression of these genes can explain the presence of 4-fold acidophilic bodies at 30 °C, compared to the

other treatments, in addition to basophilic material, and vacuolated basal compartments (López-Galindo et al., 2019).

The heat shock proteins (HSP's) are a group of functionally related proteins present in all living organisms. Among other important roles, the HSP's are involved in protein folding and unfolding, and their expression is induced by increasing temperature as well as other stresses (Wang et al., 2014). The upregulation of HSP genes constitutes the core part of the cellular heat shock response (Wang et al., 2014). We identified six members of three heat shock protein families: HSP20 (HSPB6), HSP40 (DNAJB13) and HSP70 family (HSPA9, HSPA12A, HSP70B2 and HSPA8) involved in heat stress response. HSP's as the stress-70 protein, mitochondrial (HSPA9) were highly expressed on conditions of high temperature. The family of the heat shock protein 70 is one of the most highly conserved of the HSP's. They function as molecular chaperones that act as a first defense line and mediate the refold of stress-denatured proteins, prevent the aggregation of denatured proteins and limit the cellular damage (Guzman and Conaco, 2016). HSP's protect the cell from de deleterious effects of heat and module the stress response (Castellanos-Martínez et al., 2014). The analysis of relative expression by qPCR revealed that HSPA9 gene had a high-level expression in organisms exposed to 28 °C and 30 °C in the PRE condition, while in the POST condition this gene had a high relative expression at 30 °C in comparison to 24 °C and 28 °C. Since this protein plays a role in cell proliferation, stress response and maintenance of the mitochondria, HSPA9 could be playing an important role in the preservation of mitochondria during thermal stress, which is of vital importance

The caspase-7 (CASP7) is an executioner caspase that degrades cellular components. The caspase proteins constituted the core of apoptotic machinery (Castellanos-Martínez et al., 2014). Caspases have been described in vertebrates. However, there is limited information in invertebrates such as the abalone *Haliotis diversicolor* and the mussel *M. galloprovincialis*, but there are just a few studies in cephalopods such as the common octopus *O. vulgaris* (Castellanos-Martínez et al., 2014; Romero et al., 2011). In our study, CASP7 was induced under both thermal stress and mating, presenting its higher relative expression in 30POST treatment. This expression pattern could indicate that apoptotic mechanisms have been executed in the testis of *O. maya* males under chronic thermal stress and corroborate the findings of severe tisular damage at high temperatures observed by López-Galindo et al., (2019).

The nuclear factor NF-kappa-B p100 subunit (NFKB2) is an inducible transcription factor that plays a central role in the inflammatory response and immune function, which is activated quickly by a wide group of agents and cell stress (Srikanth et al., 2017; Sun, 2017). It seems that NF-Kb is an innate immune system pathway evolutionarily conserved and present in molluscs (Castellanos-Martínez et al., 2014). NFKB2

presented significant higher relative expression under chronic thermal stress at 28 °C and 30 °C (Srikanth et al., 2017; Sun, 2017). This response confirms the inflammatory processes observed in the testis in both temperatures by López-Galindo et al., (2019). In Holstein's calves, NFKB2 has been identified as an important transcription factor that modulates the heat stress response (Srikanth et al., 2017)

The macrophage migration inhibitory factor (MIF) is a multifunctional protein which acts as a pro-inflammatory cytokine, a pituitary hormone, immunoregulator and mitogen (Anahara et al., 2008). MIF gene did not show significant differences in relative expression levels between treatments, which could be related to the multifunctional role of this protein (Anahara et al., 2008). However, a positive relationship to temperature was observed.

The Phospholipid hydroperoxide glutathione peroxidase (GPX4) protects cells against membrane lipid peroxidation and cell death (Imai et al., 2009). GPX4 gene showed a significant relative expression in organisms of 28PRE and its highest expression was observed in organisms of 30POST treatment.

The heat stress is a determinant factor that affects the physiology and reproductive performance of the organisms. Pérez-Crespo et al., (2008) mentioned that heat stress affects the sperm viability, sperm motility, reduced the fertilization capacity and survival, temporarily delays embryonic growth and promotes degeneration, causes abnormalities in the chromatin condensation, damage to DNA, RNA and protein synthesis and denatures proteins. In this study, it was possible to corroborate that thermal stress induces the expression of genes involved in the stress response to compensate the damage caused by chronic thermal stress, however, when the effect of mating is added, this expression is increased. Unfortunately, despite these compensatory mechanisms, testicular damage caused by chronic thermal stress at 30 °C is severe and directly affects the reproductive success of *O. maya* males. It is important to realize more studies that allow us to elucidate if severe testicular damage could have a gradual return to normal conditions of spermatogenesis.

3.5.2 Critical DEGs involved in spermatogenesis and spermiogenesis process in *O. maya* testis transcriptome.

Spermatogenesis is a dynamic and synchronized maturation process from germ cells to mature spermatozoa that take place in the seminiferous tubules in the testis (Shaha et al., 2010). Stringent

temporal and spatial expression of genes during both transcriptional and translational processes during protein synthesis is of fundamental importance to ensure the highly ordered processes of spermatogenesis (He et al., 2012). The goal of spermatogenesis is to produce a genetically male gamete that can fertilize an ovum ultimately produce offspring, and this process involves series of intricate, cellular, proliferative and developmental phases such as mitotic proliferation (proliferation and differentiation of spermatogonia), meiotic phase (differentiation of spermatocytes), and spermiogenesis (differentiation oh haploid germ cells from round spermatids to elongated spermatids and spermatozoa) (Dang et al., 2012; He et al., 2012; Yan et al., 2010). Protein phosphorylation is the most common post-translational protein modification in eukaryotes that controls the spermatogenesis process (Zhang et al., 2010). A protein kinase family, the testis-specific serine/threonine kinases (TSSK's) may play a role in male spermatogenesis because they are expressed mainly or specifically in the testis. Five members of the TSSK family has been identified in mouse (TSSK1, TSSK2, TSSK3, TSSK3, TSSK4 and TSSK5) (Zhang et al., 2010). Previous research revealed that TSSK2 phosphorylates several flagellar proteins in the central apparatus of the sperm axoneme, such as SPAG16 and testis-specific kinase substrate (Xu et al., 2008; Zhang et al., 2010). The TSSK2 are implied in the formation of microtubule structures during spermatogenesis and is crucial for spermatid production (Zhang et al., 2010) In this study, TSSK2 gene was identified at 30 °C in both PRE and POST conditions. The higher expression was identified in 30PRE condition. Insufficient expression of TSSK2 could interrupt spermiogenesis and results in failure of elongated spermatids, triggering male infertility. This results coincides with the lack of spermatids in the testis of octopus thermally stress and could explain the lack of parental contribution as observed by Lopez-Galindo et al., (2019).

Spermiogenesis involves three subsequent significant events: formation of the acrosome, flagellum formation and cytoplasm reorganization. Yan et al., (2010) mentioned that meiosis is unique to germ cells, and spermatogenesis is unique to male germ cell development, and this particularity demands unique genes and gene products to execute their functions. The spermatogenesis process implies the use of ~10% of the entire protein-encoding genes meanwhile spermiogenesis alone involves 500 testis-specific genes (Yan et al., 2010). There are a series of transcription factors that are important to regulate the gene expression. In the present study, we identified the transcriptional repressor ZMYND15 with significant level expression in the 30PRE treatment. ZMYND15 interacts with histone deacetylases and plays an essential role in the regulation of spatial-temporal expression of many haploid genes.

Moreover, is specifically expressed in spermatids during spermiogenesis process and is essential for normal spatio-temporal haploid gene expression. Male infertility and azoospermia have been linked to the inactivation of this gene in mice (Yan et al., 2010). In our study, ZMYND15 presents low levels of relative

gene expression in 30POST in comparison to the control. The expression pattern of this gene in invertebrates, molluscs or even cephalopods has not been described. This is the first report about the existence of this gene in cephalopods and its potential role in *O. maya* male infertility.

Another haploid gene required for male fertility during spermiogenesis is KLHL10. This gene is involved in protein ubiquitination. In mice is critical for the maturation process of spermatozoa, and is one of the essential proteins for postmeiotic spermatozoa (Yatsenko et al., 2010). KLHL10 is a member of a large BTB (Brica-brac, Tramtrack, and Broad-Complex-kelch protein superfamily, characterized by an amino-terminal BTB/POZ domain and kelch repeats at the carboxyl terminus. This protein is specifically expressed in the testis and has similar expression pattern than CUL3. Wang et al., (2006) suggested that KLHL10 interacts with CUL3 to form a CUL3-based ubiquitin E3 ligase that functions specifically in the testis to mediate protein ubiquitination during spermiogenesis. This is the first time that KLHL10 is identified in mollusks and specifically cephalopods as *O. maya*. The RNA-Seq and qPCR analysis showed that KLHL10 presented the higher expression at 30 °C in both PRE and POST condition. An increase in the expression values was observed directly proportional to the temperature. As was observed in human and mice, we can hypothesize that the increment in the expression of this gene at 30 °C affected the male fertility in *O. maya*. Disrupted spermatogenesis, degeneration of late spermatids and reduction in late spermatid number which was reported by López Galindo et al., (2019) where they observed disruption in the germ cell strata of the seminiferous tubules and did not find a parental contribution from thermally-stressed fathers.

During the spermiogenesis process, haploid germ cells are transformed into highly polarized cells with the potential for motility and fertilization (Lo et al., 2012). The sperm tail, like motile cilia and flagella of other species, contains an axoneme at its core composed of a 9+2 microtubule arrangement. The axoneme develops from a single centriole at the base of the sperm head and functions to metabolize ATP and generate microtubule sliding and motility (Lo et al., 2012; Maxwell, 1974). Defects in sperm axoneme function result in asthenospermia (Abnormal sperm motility) (Lo et al., 2012). Lo et al., (2012) identified the RABL2A gene as critically involved in sperm tail function and male fertility in mice. In this study, RABL2A, agree to RNA-Seq and qPCR analysis showed the highest expression in the 30PRE treatment. An expression increment from 24 °C to 30 °C was observed. We hypothesize that the overexpression of this gene starts with the response at 28 °C, intending to repair the damage caused to the sperm tail. However, we can attribute that the reduced parental contribution and its lack at 30 °C are directly related to the injury in sperm motility of *O. maya* males (López-Galindo et al., 2019).

Another gene involved in the sperm tail function is DNAJB13, which is a type II HSP40/DnaJ protein (Li and Liu, 2014). This gene is also known as testis spermatogenesis apoptosis-related protein expressed abundantly in mouse testis (Li and Liu, 2014). DNAJB13 was characterized in mature mouse testis and epididymal spermatozoa by Guan et al., (2010). Li and Liu (2014) confirmed the expression of DNAJB13 in the cytoplasm of spermatids and the flagella of mature spermatozoa, indicating its function in sperm motility. In our study, DNAJB13 was also highly expressed at 30 °C post copula.

According to the genes identified in response to thermal stress and spermatogenesis and spermiogenesis, it can be corroborated that the temperature significantly affects these processes carried out in the *Octopus maya* testis. These genes presented a high expression perhaps with the objective of compensating the damage caused by the rise in temperature. However, at the tissue level, these mechanisms are insufficient triggering inflammatory processes and tissue necrosis in the testis of thermally-stressed octopuses.

The increase in the expression of genes involved in spermatogenesis processes may explain the increase in the number of spermatophores observed in octopuses exposed to 28 °C and 30 °C (Lopez-Galindo et al., 2019). This expression patterns could demonstrate an effort at the reproductive level to compensate for the deleterious damage attributed to temperature.

The genes TSSK2, KLHL10, ZMYND15, RABL2A and DNAJB13 indicated that there is a harmful effect on the production of viable sperm cells, the structural conformation of sperm, capacitation and motility. This was reinforced by histological analyzes, which show the moderate to severe damage to the testis and the lost of different cell types (spermatogonia, spermatocytes, spermatids, and mature spermatozoa) in *O. maya* males exposed to temperatures above 28 °C. Chronic thermal stress generated infertility in *O. maya* which was corroborated through analysis of parental assignment, where no parental contribution of thermally-stressed parents was found.

In the other hand, as observed in *O. maya* females and embryos, the problems presented a thermal threshold at 28 °C, from which the physiological process, the reproductive performance and success, and the molecular mechanisms involved in the stress response and reproductive traits are severely affected. This study provides relevant information on the adaptive mechanisms presented in *O. maya* males against the effects of temperature. This is of vital importance, due to the predictions about the rise of sea temperatures between 2.5 to 3 °C in the western zone of the Yucatan Peninsula causing a significant reduction of the population in this area, and migration to the eastern zone of the peninsula.

Chapter 4. General discussion

In this study, *Octopus maya* males showed highly sensitive to thermal changes, as has already been observed in females, embryos and juveniles of this species (Juárez et al., 2015, 2016; Sanchez-García et al., 2017). We observed that high temperatures affect essential aspects such as growth, energy storage, activity, maintenance, reproduction and development in males of this species. It was found that the males exposed to high temperatures showed inhibition in growth from 28 °C, presenting specific growth rates and gain in weight significantly low, which has already been observed in females and embryos of this species under conditions of thermal stress (Juárez et al., 2015, 2016; Sanchez-García et al., 2017).

O. maya showed a significant increase in hemocytes at 30 °C (Figure 17). This increase is of vital importance because in mollusks in the absence of a specific immune system, the immune response is mediated by hemocytes circulating in the hemolymph and by molecular effectors that allow it to respond quickly to stressors (Castellanos-Martínez et al., 2014; Ottaviani et al., 2010). Its importance also lies in its function as a defense system against the susceptibility caused by the increase in temperature and the compromised state of health of these organisms. These mechanisms have already been reported in mollusks such as *Chamella gallina* and cephalopods such as *Eledone cirrhosa* under various stressful conditions (Malham et al., 2002; Monari et al., 2007).

Regarding energy storage, in octopuses exposed to 28 and 30 °C, we found a significant reduction in the digestive gland index (Figure 17). This reduction has been observed under starvation in *Sepia officinalis*, *Octopus vulgaris* and *Todarodes japonicas* (Castro et al., 1992). The digestive gland is a key organ to evaluate the state of health of the organisms. High temperatures affect the nutritional and health status of organisms, making them susceptible to diseases. Likewise, the digestive gland is responsible for the generation and storage of energy, which is mostly destined for the reproductive processes (Cartier et al., 2004; Castro et al., 1992). Octopuses store energy in this organ during most of their life to redirect it to the reproductive event, which involves a high energy spending. We hypothesize that under stress conditions the little energy stored is used mainly to maintenance and reproduction at the expense of growth. The digestive gland index is closely related to oxygen consumption, which showed a significant reduction at 30 °C. The increase in temperature provokes an increase in the energy demands that are essential to cover homeostasis (Sokolova et al., 2012). This metabolic suppression may indicate that *O. maya* at 28 °C in the face of moderate stress presents a compensation strategy, using diverse cellular, physiological and behavioural mechanisms that reduce the negative impact of stress (Sokolova et al.,

2012). Overall, the cellular and whole-organism compensatory processes while essential in surviving stress, are energetically costly and may have more energy flux and metabolic power from fitness-related functions such as reproduction and growth towards maintenance and repair (Sokolova et al., 2012). In this situation, the aerobic metabolism is still able to cover the energy requirements to cope with stress at least temporarily (Fusi et al., 2016; Marshall et al., 2010; Oellermann et al., 2012; Sokolova et al., 2012). In the case of organisms exposed to 30 °C, we observed a strategy of conservation where the aerobic scope disappears, and all available energy and metabolic capacity is devoted to the somatic maintenance supporting the time-limited survival of an organism but preventing growth and reproduction (Sokolova et al., 2012). Under this condition of severe stress, the energy demand is so high that both the aerobic and anaerobic mechanisms are used to cover the energy requirements for the maintenance of the organism's homeostasis temporarily.

In the case of *O. maya* males, in this study we proved, that temperature also plays a determining role in 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. Males exposed at chronic thermal stress showed a reduction in the parental contribution when the organism were exposed at 28 °C. This pattern was more evident at 30 °C where no contribution of the experimental males was found (Figure 17).

Furthermore, at the testicular level, we observed moderate damage to the testis at 28 °C. The histology showed that under this thermal condition abnormalities begin to occur in the seminiferous tubules and the cellular composition of the testis, as well as an increase in apoptotic bodies (Figure 17). Given the moderate damage caused by the thermal stress, the molecular mechanisms identified in the testis through transcriptomic analysis indicated that there is an overexpression of genes such as HSPA9, CASP7, NFKB2, GPX4 that regulate apoptosis processes, programmed cell death, inflammatory response and oxidative stress (Castellanos-Martínez et al., 2014; Guzman and Conaco, 2016; Imai et al., 2009; Srikanth et al., 2017). At molecular reproductive level at 28 °C, there was overexpression of genes such as KLHL10, TDRD1 and HTT that are involved in spermatogenesis and spermiogenesis processes (Yatsenko et al., 2010) (Figure 17 and 18). These genes are closely related to infertility in males of diverse species, mainly mouse, Holstein calves and human (Srikanth et al., 2017; Yatsenko et al., 2010). The reproductive success under these thermal conditions was affected since the parental contribution was lower than that observed in organisms under optimal thermal conditions. The results obtained under this thermal condition indicate that 28 °C represents a thermal threshold for the males of this species where the harmful effects of the thermal increase begin to be evident, partially affecting the performance and reproductive success.

At 30 °C, severe damage to the testis was observed. The histology showed a generalized disorder within the seminiferous tubules, a widening of the basement membrane, a significant increase of apoptotic bodies indicating the appearance of inflammatory processes and vacuolization. In the testis of the organism exposed to this temperature, the disappearance of various cell types (spermatozoa, spermatids and spermatocytes in its majority) was entirely evident (Figure 17). Li et al., (2015) observed in organisms of *O. tankahkeei* exposed to 32 °C during 30 min that the temperature induces injuries in the mitochondria of spermatocytes and spermatids located in the seminiferous tubules in the testis. Vacuolization may be related to the redistribution of calcium, which in turn cause a decrease in the mitochondrial membrane potential. Li et al., (2015) also observed that under thermal stress spermatogonia membranes became wrinkled, this is a direct effect over the morphology of the testis germ cells. Under this condition of stress, the expression of genes involved in apoptosis, necroptosis, stress response, oxidative stress and inflammatory response processes are observed, which indicates that at the molecular level a large number of genes are being regulated to counteract the damage caused by chronic thermal stress. At the reproductive level, the overexpression of genes involved in spermatogenesis and spermiogenesis processes such as RABL2A, TDRD1, TSSK2, KLHL10, CHD5, HTT and ZMYND15 that are important in the formation of spermatids, protein ubiquitination, sperm maturation, tail functioning, motility and structural conformation of sperm (Lo et al., 2012; Yan et al., 2010; Yatsenko et al., 2010; Zhang et al., 2010) (Figure 17 and 18). The temperature severely affected the reproductive success of males exposed to 30 °C. Despite various molecular mechanisms to try to compensate for the damage caused by temperature, the males cannot contribute to the progeny.

In general, we can see that *O. maya* has a series of physiological, metabolic and molecular mechanisms that allow it to respond to chronic thermal stress. However, these mechanisms become inefficient when the stress overpasses its thermal limits (Figure 17).

The findings obtained in this study have an important relevance at an environmental level, since Enriquez et al., (2013) and Saldivar-Lucio et al., (2015) have made predictions about the rise of sea temperatures between 2.5 to 3 °C in the zone in the Yucatan Peninsula where upwelling has no effect. According to the thermal limits already established for females (27 °C), embryos (26 °C) and now males (28 °C) could seriously affect the population dynamics of *O. maya* on the continental shelf of the Yucatan Peninsula. We hypothesized that the population could be drastically reduced or massive migrations could occur in areas with lower temperatures or near to the thermal optimum.

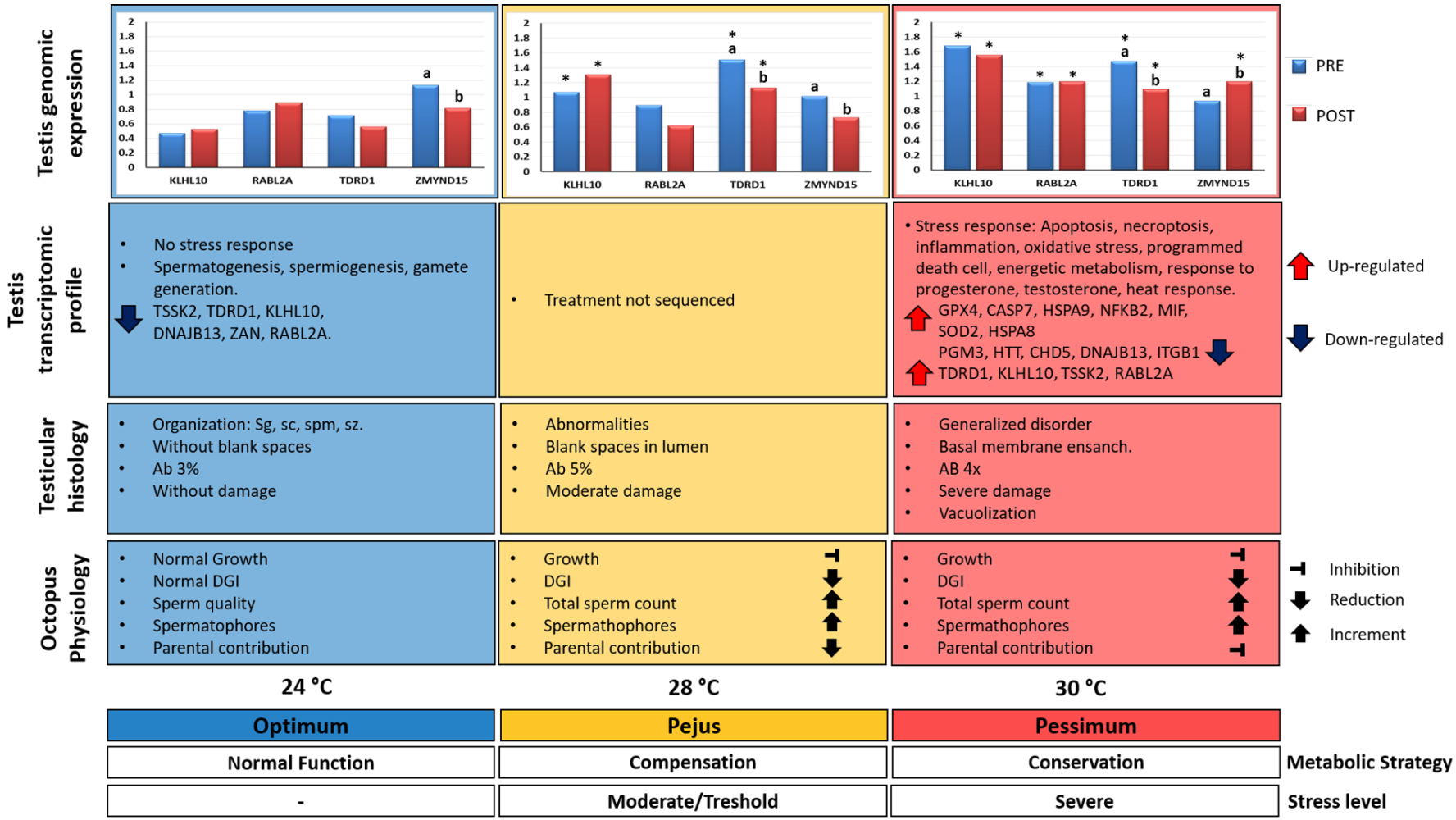

Figure 17. Schematic integration of the physiological and molecular mechanisms involved in response to the chronic thermal stress of *O. maya* males. The temperature of 24 °C represents the optimal thermal condition for this species. No differences between PRE (before mating) and POST (after mating) conditions were observed in octopus physiology and testicular histology. The genomic expression data are expressed in normalized relative quantities of expression (fold). Different letters indicate only significant differences (P<0.05) between PRE and POST conditions of the same temperature. Asterisk indicates significant differences between temperatures of the same condition.

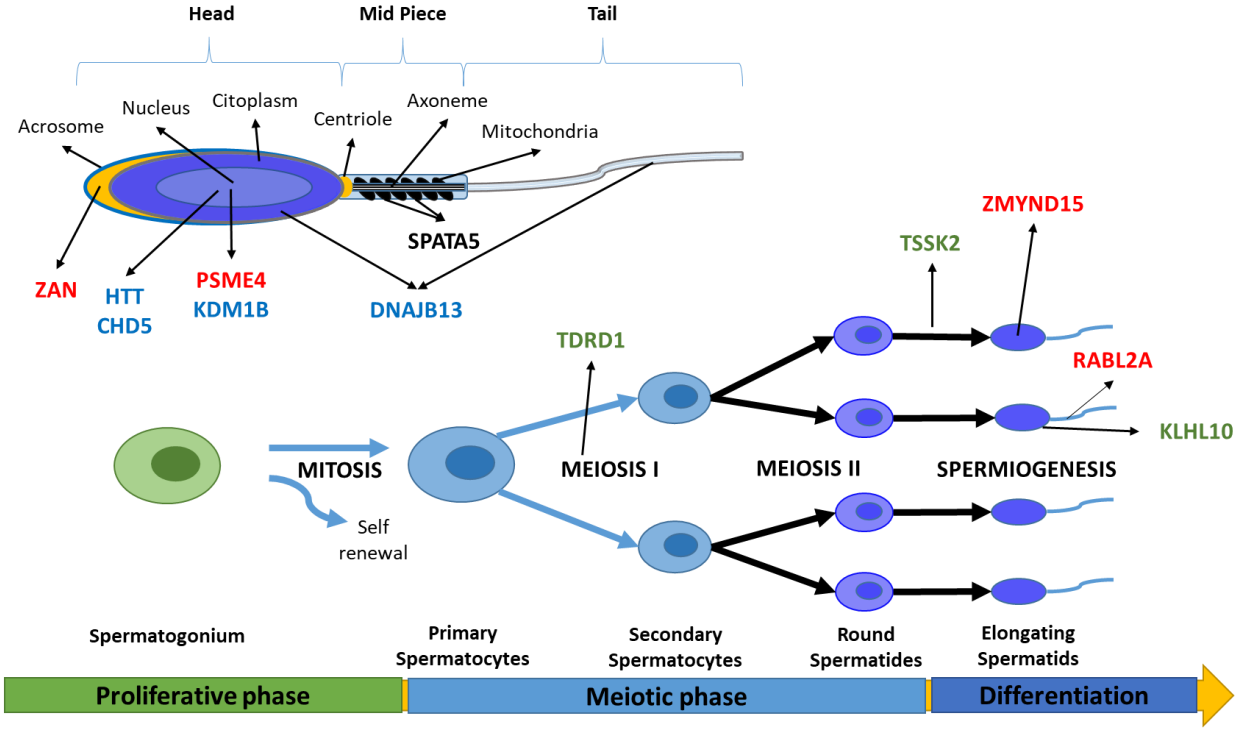

Figure 18. Schematic representation with genes significantly up-regulated under conditions of chronic thermal stress at 30 °C in *O. maya* males, which play an important role in male fertility during the processes of spermatogenesis and spermiogenesis in the testis. The scheme shows the three phases involved in the spermatogenesis process, as well as, the type of cellular division and the different germinal cells produced during the process. Genes highly expressed in 30PRE treatment are shown in red; 30POST in blue and overexpressed in both treatments in green. The genes identified by the transcriptome analysis has very important functions during the spermatogenesis process. ZMYND15 (Zinc finger MYND domain-containing protein 15) regulate the spatial-temporal expression of haploid genes; KLHL10 (Kelch-like protein 10) involved in protein ubiquitination during spermiogenesis; RABL2A (Rab-like protein 2A) in sperm tail function; DNAJB13 (DnaJ homolog subfamily B member 13) in sperm motility; ZAN (Zonadhesin) in signaling and gamete recognition; HTT (Huntingtin) protein synthesis and chromatin remodeling; CHD5 (Chromodomain helicase-DNA-binding protein 5) condensation of chromatin; PSME4 (Proteasome activator complex subunit 4) participate actively in the exchange of histones; KDM1B (Lysine-specific histone demethylase 1B) regulate histone lysine methylation, an epigenetic mark that regulates gene expression and chromatin function; TDRD1 (Tudor domain-containing protein 1) maintain germ cell integrity; and TSSK2 (Testis-specific serine/threonine-protein kinase 2) required for the transformation of a ring-shaped structure around the base of the flagellum originating from the chromatoid body. SPATA5 (black) is an important gene which is involved in morphological and functional mitochondrial transformations during spermatogenesis. This gene was down-regulated at 30 °C.

Chapter 5. General conclusions

Under optimal conditions of temperature, the mating induces the expression of genes that allow to carry out the normal processes of spermatogenesis and generation of germ cells in the testis of *O. maya*. This expression pattern in the testis coupled with the physiological condition favour the performance and reproductive success of the *Octopus maya* males.

The high temperatures affect the physiology, growth and reproduction of males of the species *O. maya.*

High temperatures cause severe damage to the morphology of the testis, inflammation and a significant deterioration of the different cell types, which implies that the reproductive effort to the production of a higher number of spermatophores is insufficient to leave offspring. Also, this damage induces a stress response through the expression of genes involved in processes of apoptosis, necroptosis, inflammation and programmed cell death. At reproductive level, the molecular mechanisms involved in the processes of gamete generation, spermatogenesis and spermiogenesis seek in some way to compensate for the damage caused by temperature, by inducing the expression of genes such as TSSK2, TDRD1, KLHL10, ZMYND15, among others, to increase the quality of the sperm. However, this strategy does not reduce infertility caused by severe deterioration of the testis.

In *O. maya* males temperatures above 28 °C have a deleterious effect on performance, reproductive success, testicular quality and molecular mechanisms that regulate reproduction and sperm production. This temperature determines the thermal threshold in the *O. maya* males.

Bibliographic references

Akmal, M., Aulanni'am, A., Widodo, M. A., Sumitro, S. B., Purnomo, B. B., and Widodo (2016). The important role of protamine in spermatogenesis and quality of sperm: A mini review. *Asian Pacific J. Reprod.* 5, 357–360. doi:10.1016/j.apjr.2016.07.013.

Anahara, R., Toyama, Y., and Mori, C. (2008). Review of the histological effects of the anti-androgen, flutamide, on mouse testis. *Reprod. Toxicol.* 25, 139–143. doi:10.1016/j.reprotox.2007.12.003.

Anderson, R. C., Wood, J. B., and Byrne, R. A. (2002). Octopus Senescence: The Beginning of the End. J. Appl. Anim. Welf. Sci. 5, 275–283. doi:10.1207/S15327604JAWS0504.

André, J., Pecl, G. T., Semmens, J. M., and Grist, E. P. M. (2008). Early life-history processes in benthic octopus: Relationships between temperature, feeding, food conversion, and growth in juvenile *Octopus pallidus*. *J. Exp. Mar. Bio. Ecol.* 354, 81–92. doi:10.1016/j.jembe.2007.10.008.

Andrews, P. L. R., Darmaillacq, A. S., Dennison, N., Gleadall, I. G., Hawkins, P., Messenger, J. B., et al. (2013). The identification and management of pain, suffering and distress in cephalopods, including anaesthesia, analgesia and humane killing. *J. Exp. Mar. Bio. Ecol.* 447, 460–64. doi:10.1016/j.jembe.2013.02.010.

Angeles-Gonzalez, L. E., Calva, R., Santos-Valencia, J., Avila-Poveda, O. H., Olivares, A., Diaz, F., et al. (2017). Temperature modulates spatio-temporal variability of the functional reproductive maturation of *Octopus maya* (Cephalopoda) on the shelf of the Yucatan Peninsula, Mexico. *J. Molluscan Stud.* 83, 280–288. doi:10.1093/mollus/eyx013.

Arreguín-Sánchez, F. (2000). Octopus – red grouper interaction in the exploited ecosystem of the northern continental shelf of Yucatan , Mexico. *Ecol. Modell.* 129, 119–129. doi:doi.org/10.1016/S0304-3800(00)00218-0.

Artigaud, S., Richard, J., Thorne, M. A. S., Lavaud, R., Flye-Sainte-Marie, J., Jean, F., et al. (2015). Deciphering the molecular adaptation of the king scallop (*Pecten maximus*) to heat stress using transcriptomics and proteomics. *BMC Genomics* 16, 1–14. doi:10.1186/s12864-015-2132-x.

Avila-Poveda, O. H., Colin-Flores, R. F., and Rosas, C. (2009). Gonad development during the early life of *Octopus maya* (mollusca: cephalopoda). *Biol. Bull.* 216, 94–102.

Avila-Poveda, O. H., Koueta, N., Benítez-Villalobos, F., Santos-Valencia, J., and Rosas, C. (2016). Reproductive traits of *Octopus maya* (Cephalopoda: Octopoda) with implications for fisheries management. *Molluscan Res.* 36, 29–44. doi:10.1080/13235818.2015.1072912.

Avila-Poveda, O. H., Montes-Pérez, R. C., Koueta, N., Benítez-Villalobos, F., Ramírez-Pérez, J. S., Jimenez-Gutierrez, L. R., et al. (2015). Seasonal changes of progesterone and testosterone concentrations throughout gonad maturation stages of the Mexican octopus, *Octopus maya* (Octopodidae: Octopus). *Molluscan Res.* 35, 161–172. doi:10.1080/13235818.2015.1045055.

Barnes, R. 1987. Invertebrate Zoology. Orlando, Florida: Dryden Press.

Birkhead, T. R., and Møller, A. P. (1998). *Sperm Competition and Sexual Selection*. San Diego, California: Academia Press doi:https://doi.org/10.1016/B978-0-12-100543-6.X5022-3.

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi:10.1093/bioinformatics/btu170.

Bozinovic, F., and Pörtner, H. O. (2015). Physiological ecology meets climate change. *Ecol. Evol.* 5, 1025–1030. doi:10.1002/ece3.1403.

Brusca, R, C. y Brusca, G, J. 2003. Invertebrates. Second edition. pp. 754-759.

Caamal-Monsreal, C., Uriarte, I., Farias, A., Díaz, F., Sánchez, A., Re, D., et al. (2016). Effects of temperature on embryo development and metabolism of *O. maya*. *Aquaculture* 451, 156–162. doi:10.1016/j.aquaculture.2015.09.011.

Cabrera, M. A., Miranda, J. R., Salas, S., Hernández, D. F., and López, S. (2012). Population Structure Analysis of Red Octopus (*Octopus maya*) in the Yucatan Peninsula , Mexico. *Proc. Gulf Caribb. Fish. Inst.* 64.

Camacho-Sanchez, M., Burraco, P., Gomez-Mestre, I., and Leonard, J. A. (2013). Preservation of RNA and DNA from mammal samples under field conditions. *Mol. Ecol. Resour.* 13, 663–673. doi:10.1111/1755-0998.12108.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., et al. (2009). BLAST+: Architecture and applications. *BMC Bioinformatics* 10, 1–9. doi:10.1186/1471-2105-10-421.

Cartier, S., Pellerin, J., Fournier, M., Tamigneaux, E., Girault, L., and Lemaire, N. (2004). Use of an index based on the blue mussel (*Mytilus edulis* and *Mytilus trossulus*) digestive gland weight to assess the nutritional quality of mussel farm sites. *Aquaculture* 241, 633–654. doi:10.1016/j.aquaculture.2004.08.015.

Caruana, N. J., Cooke, I. R., Faou, P., Finn, J., Hall, N. E., Norman, M., et al. (2016). A combined proteomic and transcriptomic analysis of slime secreted by the southern bottletail squid, *Sepiadarium austrinum* (Cephalopoda). *J. Proteomics* 148, 170–182. doi:10.1016/j.jprot.2016.07.026.

Castellanos-Martínez, S., Arteta, D., Catarino, S., and Gestal, C. (2014). De novo transcriptome sequencing of the *Octopus vulgaris* hemocytes using illumina RNA-Seq technology: Response to the infection by the gastrointestinal parasite Aggregata octopiana. *PLoS One* 9. doi:10.1371/journal.pone.0107873.

Castro, B. G., Garrido, J. L., and Sotelo, C. G. (1992). Changes in composition of digestive gland and mantle muscle of the cuttlefish *Sepia officinalis* during starvation. *Mar. Biol.* 114, 11–20. doi:10.1007/BF00350851.

Cerezo Valverde, J., Hernández, M. D., Aguado-Giménez, F., and García García, B. (2008). Growth, feed efficiency and condition of common octopus (*Octopus vulgaris*) fed on two formulated moist diets. *Aquaculture* 275, 266–273. doi:10.1016/j.aquaculture.2008.01.012.

Conesa, A., and Götz, S. (2008). Blast2GO: A comprehensive suite for functional analysis in plant genomics. *Int. J. Plant Genomics* 2008. doi:10.1155/2008/619832.

Cornet, V., Henry, J., Corre, E., Le Corguille, G., Zanuttini, B., and Zatylny-Gaudin, C. (2014). Dual role of the cuttlefish salivary proteome in defense and predation. *J. Proteomics* 108, 209–222. doi:10.1016/j.jprot.2014.05.019.

Dang, R., Zhu, J. Q., Tan, F. Q., Wang, W., Zhou, H., and Yang, W. X. (2012). Molecular characterization of a KIF3B-like kinesin gene in the testis of *Octopus tankahkeei* (cephalopoda, octopus). *Mol. Biol. Rep.* 39, 5589–5598. doi:10.1007/s11033-011-1363-4.

Deutsch, C., Ferrel, A., Seibel, B., Pörtner, H.-O., and Huey, R. B. (2015). Constraint on Marine Habitats. *Science (80-.).* 348, 1132–1136.

Doubleday, Z. A., Prowse, T. A. A., Arkhipkin, A., Pierce, G. J., Semmens, J., Steer, M., et al. (2016). Global proliferation of cephalopods. *Curr. Biol.* 26, R387–R407. doi:10.1016/j.cub.2016.04.002.

Ekblom, R., and Galindo, J. (2011). Applications of next generation sequencing in molecular ecology of non-model organisms. *Heredity (Edinb).* 107, 1–15. doi:10.1038/hdy.2010.152.

Enriquez, C., Mariño-Tapia, I. J., and Herrera-Silveira, J. A. (2010). Dispersion in the Yucatan coastal zone: Implications for red tide events. *Cont. Shelf Res.* 30, 127–137. doi:10.1016/j.csr.2009.10.005.

Enriquez, C., Mariño-Tapia, I., Jeronimo, G., and Capurro-Filograsso, L. (2013). Thermohaline processes in a tropical coastal zone. *Cont. Shelf Res.* 69, 101–109. doi:10.1016/j.csr.2013.08.018.

Estefanell, J., Socorro, J., Afonso, J. M., Roo, J., Fernandez-Palacios, H., and Izquierdo, M. S. (2011). Evaluation of two anaesthetic agents and the passive integrated transponder tagging system in *Octopus vulgaris* (Cuvier 1797). *Aquac. Res.* 42, 399–406. doi:10.1111/j.1365-2109.2010.02634.x.

Excoffier, L., Laval, G., and Schneider, S. (2005). Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evol. Bioinforma.* 1, 47–50.

Fusi, M., Cannicci, S., Daffonchio, D., Mostert, B., Pörtner, H. O., and Giomi, F. (2016). The trade-off between heat tolerance and metabolic cost drives the bimodal life strategy at the air-water interface. *Sci. Rep.* 6, 1–8. doi:10.1038/srep19158.

Gamboa-Álvarez, M. Á., López-Rocha, J. A., and Poot-López, G. R. (2015). Spatial Analysis of the Abundance and Catchability of the Red Octopus *Octopus maya* (Voss and Solís-Ramírez, 1966) on the Continental Shelf of the Yucatan Peninsula, Mexico. *J. Shellfish Res.* 34, 481–492. doi:10.2983/035.034.0232.

Gleadall, I. G. (2013). The effects of prospective anaesthetic substances on cephalopods: Summary of original data and a brief review of studies over the last two decades. *J. Exp. Mar. Bio. Ecol.* 447, 23–30. doi:10.1016/j.jembe.2013.02.008.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* 29, 644–652. doi:10.1038/nbt.1883.

Guan, J., Ekwurtzel, E., Kvist, U., Hultenby, K., and Yuan, L. (2010). DNAJB13 is a radial spoke protein of mouse "9+2" axoneme. *Reprod. Domest. Anim.* 45, 992–996. doi:10.1111/j.1439-0531.2009.01473.x.

Guerra, A. 1992. Fauna ibérica. Mollusca: Cephalopoda. Museo Nacional de ciencias Naturales, Consejo Superior de Investigaciones científicas. Madrid. 327 pp.

Guzman, C., and Conaco, C. (2016). Gene expression dynamics accompanying the sponge thermal stress response. *PLoS One* 11, 1–15. doi:10.1371/journal.pone.0165368.

Hanlon, R.T., Forsythe, J.W., 1985. Advances in the laboratory culture of Octopuses for biomedical research. Laboratory Animal Science 35, 40.

He, L., Wang, Q., Jin, X., Wang, Y., Chen, L., Liu, L., et al. (2012). Transcriptome profiling of testis during sexual maturation stages in *Eriocheir sinensis* using Illumina sequencing. *PLoS One* 7. doi:10.1371/journal.pone.0033735.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* 8, R19. doi:10.1186/gb-2007-8-2-r19.

Hinojosa Ochoa, B. M. (2018). Carta Nacional Pesquera (Tercera sección). *Secr. Agric. Ganad. Desarro. Rural. Pesca y Aliment.*, 268. Available at: http://www.dof.gob.mx/nota_detalle.php?codigo=5525712&fecha=11/06/2018&print=true.

Hirohashi, N., and Iwata, Y. (2016). The different types of sperm morphology and behavior within a single species. *Commun. Integr. Biol.* 6, e26729. doi:10.4161/cib.26729.

Hoving, H.J., Lipinski, M.R., Videler, J.J. y Bolstad, K.S. 2010. Sperm storage and mating in the deep-sea squid *Taningia danae* Joubin, 1931(Oegopsida: Octopoteuthidae). Mar Biol. 157: 393-400.

Imai, H., Hakkaku, N., Iwamoto, R., Suzuki, J., Suzuki, T., Tajima, Y., et al. (2009). Depletion of selenoprotein GPx4 in spermatocytes causes male infertility in mice. *J. Biol. Chem.* 284, 32522–32532. doi:10.1074/jbc.M109.016139.

Iwata, Y., Munehara, H., and Sakurai, Y. (2005). Dependence of paternity rates on alternative reproductive behaviors in the squid *Loligo bleekeri*. *Mar. Ecol. Prog. Ser.* 298, 219–228.

Jones, A. G. (2005). 2 . 0 : a computer program for the reconstruction of parental genotypes from half-sib progeny arrays with known or unknown parents. *Mol. Ecol. Notes* 5, 708–711. doi:10.1111/j.1471-8286.2005.01029.x.

Jones, O. R., and Wang, J. (2010). COLONY : a program for parentage and sibship inference from multilocus genotype data. *Mol. Ecol. Resour.* 10, 551–555. doi:10.1111/j.1755-0998.2009.02787.x.

Juárez, O. E., Enríquez, L., Camarena-Rosales, F., Arena, L., Galindo-Sánchez, C. E., Lafarga-De la Cruz, F., et al. (2018). Genetic monitoring of the Mexican four-eyed octopus *Octopus maya* population: New insights and perspectives for the fishery management. *Fish. Res.* 206, 109–114. doi:10.1016/j.fishres.2018.05.002.

Juárez, O. E., Galindo-Sánchez, C. E., Díaz, F., Re, D., Sánchez-García, A. M., Camaal-Monsreal, C., et al. (2015). Is temperature conditioning *Octopus maya* fitness? *J. Exp. Mar. Bio. Ecol.* 467, 71–76. doi:10.1016/j.jembe.2015.02.020.

Juárez, O. E., Hau, V., Caamal-Monsreal, C., Galindo-Sánchez, C. E., Díaz, F., Re, D., et al. (2016). Effect of maternal temperature stress before spawning over the energetic balance of *Octopus maya* juveniles exposed to a gradual temperature change. *J. Exp. Mar. Bio. Ecol.* 474, 39–45. doi:10.1016/j.jembe.2015.10.002.

Juárez, O. E., Rosas, C., and Arena-Ortiz, M. L. (2012). Phylogenetic relationships of *Octopus maya* revealed by mtDNA sequences Relaciones. *Ciencias Mar.* 38, 563–575. doi:10.7773/cm.v38i3.1962.

Juárez, O. E., Rosas, C., Arena, L., Camarena, F., McKeown, N., and Shaw, P. W. (2013). Characterization of microsatellite loci developed for the Mexican four-eyed octopus *Octopus maya*. *Conserv. Genet. Resour.* 5, 803–805. doi:10.1007/s12686-013-9912-x.

Kanehisa, M., and Goto, S. (2000). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* 28, 27–30. doi:10.1093/nar/27.1.29.

Kim, B. M., Kim, K., Choi, I. Y., and Rhee, J. S. (2017). Transcriptome response of the Pacific oyster, *Crassostrea gigas* susceptible to thermal stress: A comparison with the response of tolerant oyster. *Mol. Cell. Toxicol.* 13, 105–113. doi:10.1007/s13273-017-0011-z.

Koressaar, T., and Remm, M. (2007). Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23, 1289–1291. doi:10.1093/bioinformatics/btm091.

Krstulovic-Sifner, S., and Vrgoc, N. (2009). Reproductive cycle and sexual maturation of the musky octopus *Eledone moschata* (Cephalopoda : Octopodidae) in the northern and central Adriatic Sea. *Sci. Mar.* 73, 439–447. doi:10.3989/scimar.2009.73n3439.

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi:10.1038/nmeth.1923.

Larson, S., Ramsay, C., and Cosgrove, J. A. (2015). Multiple Paternity and Preliminary Population Genetics of Giant Pacific Octopuses, *Enteroctopus dofleini*, in Oregon, Washington and the Southeast Coast of Vancouver Island, BC. *Diversity* 7, 195–205. doi:10.3390/d7020195.

Li, B., and Dewey, C. N. (2011). RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12. doi:10.1186/1471-2105-12-323.

Li, G. L., and Qian, H. (2017). Transcriptome using Illumina sequencing reveals the traits of spermatogenesis and developing testes in *Eriocheir sinensis*. *PLoS One* 12, 1–16. doi:10.1371/journal.pone.0172478.

Li, W., and Liu, G. (2014). DNAJB13, a type II HSP40 family member, localizes to the spermatids and spermatozoa during mouse spermatogenesis. *BMC Dev. Biol.* 14, 1–7. doi:10.1186/s12861-014-0038-5.

Lim, H. J., Kim, B. M., Hwang, I. J., Lee, J. S., Choi, I. Y., Kim, Y. J., et al. (2016). Thermal stress induces a distinct transcriptome profile in the Pacific oyster *Crassostrea gigas*. *Comp. Biochem. Physiol. - Part D Genomics Proteomics* 19, 62–70. doi:10.1016/j.cbd.2016.06.006.

Liu, C., Zhao, F., Yan, J., Liu, C., Liu, S., and Chen, S. (2016). Transcriptome sequencing and de novo assembly of golden cuttlefish *Sepia esculenta* Hoyle. *Int. J. Mol. Sci.* 17. doi:10.3390/ijms17101749.

Lo, J. C. Y., Jamsai, D., O'Connor, A. E., Borg, C., Clark, B. J., Whisstock, J. C., et al. (2012). RAB-Like 2 Has an Essential Role in Male Fertility, Sperm Intra-Flagellar Transport, and Tail Assembly. *PLoS Genet.* 8. doi:10.1371/journal.pgen.1002969.

Long, L. L., Han, Y. L., Sheng, Z., Du, C., Wang, Y. F., and Zhu, J. Q. (2015). Expression analysis of HSP70 in the testis of *Octopus tankahkeei* under thermal stress. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* 187, 150–159. doi:10.1016/j.cbpa.2015.05.022.

López-Galindo, L., Galindo-Sánchez, C., Olivares, A., Avila-Poveda, O. H., Díaz, F., Juárez, O. E., et al. (2019). Reproductive performance of *Octopus maya* males conditioned by thermal stress. *Ecol. Indic.* 96, 437–447. doi:10.1016/j.ecolind.2018.09.036.

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 1–21. doi:10.1186/s13059-014-0550-8.

Lü, Z., Liu, W., Liu, L., Shi, H., Ping, H., Wang, T., et al. (2016). De novo assembly and comparison of the ovarian transcriptomes of the common Chinese cuttlefish (*Sepiella japonica*) with different gonadal development. *Genomics Data* 7, 155–158. doi:10.1016/j.gdata.2015.12.011.

Malham, S. K., Lacoste, A., Gélébart, F., Cueff, A., and Poulet, S. A. (2002). A first insight into stress-induced neuroendocrine and immune changes in the octopus *Eledone cirrhosa*. *Aquat. Living Resour.* 15, 187–192.

Mangold, K. 1983. *Octopus vulgaris*. In: P. R. Boyle (De). Cephalopod Life Cycles Vol. I.Species Accounts. Academic ress Inc. (London), pp. 335-365.

Marshall, D. J., McQuaid, C. D., and Williams, G. a (2010). Non-climatic thermal adaptation: implications for species' responses to climate warming. *Biol. Lett.* 7, 160. doi:10.1098/rsbl.2010.0233.

Mather, J. A., and Anderson, R. C. (2007). Ethics and invertebrates: A cephalopod perspective. *Dis. Aquat. Organ.* 75, 119–129. doi:10.3354/dao075119.

Maxwell, W. L. (1974). Spermiogenesis of *Eledone cirrhosa* Lamarck (Cephalopoda, Octopoda). *Proc. R. Soc. London - Biol. Sci.* 186, 181–190. doi:10.1098/rspb.1974.0045.

Moltschaniwskyj, N. A., Hall, K., Lipinski, M. R., Marian, J. E. A. R., Nishiguchi, M., Sakai, M., et al. (2007). Ethical and welfare considerations when using cephalopods as experimental animals. *Rev. Fish Biol. Fish.* 17, 455–476. doi:10.1007/s11160-007-9056-8.

Monari, M., Matozzo, V., Foschi, J., Cattani, O., Serrazanetti, G. P., and Marin, M. G. (2007). Effects of high temperatures on functional responses of haemocytes in the clam *Chamelea gallina*. *Fish Shelfish Inmunol.* 22, 98–114. doi:10.1016/j.fsi.2006.03.016.

Nguyen, K. D. T., Morley, S. A., Lai, C., Clark, M. S., Tan, K. S., Bates, A. E., et al. (2011). Upper Temperature Limits of Tropical Marine Ectotherms : Global Warming Implications. *PLoS One* 6, e29340. doi:10.1371/journal.pone.0029340.

Noyola, J., Caamal-Monsreal, C., Díaz, F., Re, D., Sánchez, A., and Rosas, C. (2013a). Thermopreference, tolerance and metabolic rate of early stages juvenile *Octopus maya* acclimated to different temperatures. *J. Therm. Biol.* 38, 14–19. doi:10.1016/j.jtherbio.2012.09.001.

Noyola, J., Mascaró, M., Caamal-Monsreal, C., Noreña-Barroso, E., Díaz, F., Re, D., et al. (2013b). Effect of temperature on energetic balance and fatty acid composition of early juveniles of *Octopus maya*. *J. Exp. Mar. Bio. Ecol.* 445, 156–165. doi:10.1016/j.jembe.2013.04.008.

Noyola, R. J., Mascaro, M., Díaz, F., Denisse Re, A., Sánchez-Zamora, A., Caamal-Monsreal, C., et al. (2015). Thermal biology of prey (*Melongena corona bispinosa, Strombus pugilis, Callinectes similis, Libinia dubia*) and predators (*Ocyurus chrysurus, Centropomus undecimalis*) of *Octopus maya* from the Yucatan Peninsula. *J. Therm. Biol.* 53, 151–161. doi:10.1016/j.jtherbio.2015.11.001.

O'Donnell, L. (2014). Mechanisms of spermiogenesis and spermiation and how they are disturbed. *Spermatogenesis* 4, e979623. doi:10.4161/21565562.2014.979623.

Oellermann, M., Pörtner, H. O., and Mark, F. C. (2012). Mitochondrial dynamics underlying thermal plasticity of cuttlefish (*Sepia officinalis*) hearts. *J. Exp. Biol.* 215, 2992–3000. doi:10.1242/jeb.068163.

Olivares, A., Avila-Poveda, O. H., Leyton, V., Zuñiga, O., Rosas, C., and Northland-Leppe, I. (2017). Oviducal glands throughout the gonad development stages: A case study of *Octopus mimus* (cephalopoda). *Molluscan Res.* 37, 229–241. doi:10.1080/13235818.2017.1334275.

Ottaviani, E., Franchini, A., and Malagoli, D. (2010). Inflammatory Response in Molluscs: Cross-Taxa and Evolutionary Considerations. *Curr. Pharm. Des.* 16, 4160–4165. doi:10.2174/138161210794519084.

Parker, G. A. (2016). The evolution of expenditure on testes. *Jorunal Zool.* 298, 3–19. doi:10.1111/jzo.12297.

Pascual, C., Hernández Franyutti, A., and Gómez Guzmán, I. (2011). Caracterización Morfológica de los Hemocitos del Pulpo Rojo, *Octopus maya*. *64th Gulf Caribb. Fish. Inst.*, 490–494.

Pérez-Crespo, M., Pintado, B., and Gutiérrez-Adán, A. (2008). Scrotal Heat Stress Effects on Sperm Viability, Sperm DNA Integrity, and the Offspring Sex Ratio in Mice. *Mol. Reprod. Dev.* 75, 40–47. doi:10.1002/mrd.

Peterson, J. S., Timmons, A. K., Mondragon, A. A., and McCall, K. (2015). *The end of the beginning: Cell death in the Germline*. 1st ed. Elsevier Inc. doi:10.1016/bs.ctdb.2015.07.025.

Piasečná, K., Ponťová, A., Tejedo, M., and Gvoždík, L. (2015). Thermoregulatory strategies in an aquatic ectotherm from thermally-constrained habitats: An evaluation of current approaches. *J. Therm. Biol.* 52, 97–107. doi:10.1016/j.jtherbio.2015.06.007.

Pigliucci, M. (1996). How organisms respond to environmental changes: from phenotypes to molecules (and vice versa). *Trends Ecol. Evol.* 11, 168–173. doi:10.1016/0169-5347(96)10008-2.

Quinteiro, J., Baibai, T., Oukhattar, L., Soukri, A., Seixas, P., and Rey-Méndez, M. (2011). Multiple paternity in the common octopus *Octopus vulgaris* (Cuvier, 1797), as revealed by microsatellite DNA analysis. *Molluscan Res.* 31, 15–20.

Rocha, F. 2003. Biología, Ecología, Cultivo y Pesquerías De Cefalópodos. Vigo, España: Instituto de Investigaciones Marinas (Consejo Superior de Investigaciones Científicas). 1-207.

Rodrigues, M., Guerra, Á., and Troncoso, J. S. (2011). Reproduction of the Atlantic bobtail squid *Sepiola atlantica* (Cephalopoda : Sepiolidae) in northwest Spain. *Invertebr. Biol.* 131, 30–39. doi:10.1111/j.1744-7410.2011.00253.x.

Romero, A., Estévez-Calvar, N., Dios, S., Figueras, A., and Novoa, B. (2011). New insights into the apoptotic process in mollusks: Characterization of caspase genes in *Mytilus galloprovincialis*. *PLoS One* 6. doi:10.1371/journal.pone.0017003.

Rosa, R., Gonzalez, L., Dierssen, H. M., and Seibel, B. A. (2012). Environmental determinants of latitudinal size-trends in cephalopods. *Mar. Ecol. Prog. Ser.* 464, 153–165. doi:10.3354/meps09822.

Rosas, C., Gallardo, P., Mascaró, M., Caamal-monsreal, C., and Pascual, C. (2014). "*Octopus maya*," in *Cephalopod culture*, eds. J. Iglesias, L. Fuentes, and R. Villanueva (Springer Netherlands), 383–396.

Rosas, C., Tut, J., Baeza, J., Sánchez, A., Sosa, V., Pascual, C., et al. (2008). Effect of type of binder on growth, digestibility, and energetic balance of *Octopus maya*. *Aquaculture* 275, 291–297. doi:10.1016/J.AQUACULTURE.2008.01.015.

Salazar, K. A., Joffe, N. R., Dinguirard, N., Houde, P., and Castillo, M. G. (2015). Transcriptome analysis of the white body of the squid *Euprymna tasmanica* with emphasis on immune and hematopoietic gene discovery. *PLoS One* 10, 1–20. doi:10.1371/journal.pone.0119949.

Saldívar-Lucio, R., Salvadeo, C., Monte-Luna, P. Del, Arreguín-Sánchez, F., Villalobos, H., Lluch-Belda, D., et al. (2015). Historical patterns and predicted thermal scenarios in Mexican seas. *Rev. Biol. Mar. y Ocenaografía* 50, 331–345. doi:10.4067/S0718-19572015000300011.

Sanchez-García, A., Rodríguez-Fuentes, G., Díaz, F., Galindo-Sánchez, C. E., Ortega, K., Mascaró, M., et al. (2017). Thermal sensitivity of *O . maya* embryos as a tool for monitoring the effects of environmental warming in the Southern of Gulf of Mexico. *Ecol. Indic.* 72, 574–585.

Shaha, C., Tripathi, R., and Prasad Mishra, D. (2010). Male germ cell apoptosis: Regulation and biology. *Philos. Trans. R. Soc. B Biol. Sci.* 365, 1501–1515. doi:10.1098/rstb.2009.0124.

Sheng, K., Liang, X., Huang, S., and Xu, W. (2014). The role of histone ubiquitination during spermatogenesis. *Biomed Res. Int.* 2014. doi:10.1155/2014/870695.

Shiel, B. P., Hall, N. E., Cooke, I. R., Robinson, N. A., and Strugnell, J. M. (2014). De Novo Characterisation of the Greenlip Abalone Transcriptome (*Haliotis laevigata*) with a Focus on the Heat Shock Protein 70 (HSP70) Family. *Mar. Biotechnol.* 17, 23–32. doi:10.1007/s10126-014-9591-y.

Sivashanthini, K., Thulasitha, W. S., and Charles, G. A. (2010). Reproductive characteristics of squid Se*pioteuthis lessoniana* (Lesson, 1830) from the Northern coast of Sri Lanka. *J. Fish. Aquat. Sci.* 5, 12–22.

Sokolova, I. M., Frederich, M., Bagwe, R., Lannig, G., and Sukhotin, A. A. (2012). Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Mar. Environ. Res.* 79, 1–15. doi:10.1016/j.marenvres.2012.04.003.

Solís-Ramírez, J. M. (1967). Aspectos biológicos del pulpo *Octopus maya* Voss y Solís. *Inst. Nac. Investig. Biológico Pesq.* 18, 1–90.

Somero, G. N. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers.' *J. Exp. Biol.* 213, 912–920. doi:10.1242/jeb.037473.

Sousounis, K., Ogura, A., and Tsonis, P. A. (2013). Transcriptome Analysis of Nautilus and Pygmy Squid Developing Eye Provides Insights in Lens and Eye Evolution. *PLoS One* 8, 1–13. doi:10.1371/journal.pone.0078054.

Squires, Z. E., Wong, B. B. M., Norman, M. D., and Stuart-fox, D. (2014). Multiple paternity but no evidence of biased sperm use in female dumpling squid *Euprymna tasmanica*. *Mar. Ecol. Prog. Ser.* 511, 93–103. doi:10.3354/meps10898.

Srikanth, K., Lee, E., Kwan, A., Lim, Y., Lee, J., Jang, G., et al. (2017). Transcriptome analysis and identification of significantly differentially expressed genes in Holstein calves subjected to severe thermal stress. *Int. J. Biometeorol.* 61, 1993–2008. doi:10.1007/s00484-017-1392-3.

Sun, S. C. (2017). The non-canonical NF-κB pathway in immunity and inflammation. *Nat. Rev. Immunol.* 17, 545–558. doi:10.1038/nri.2017.52.

Takahara, H., Kidokoro, H., and Sakurai, Y. (2016). High temperatures may halve the lifespan of the Japanese flying squid, *Todarodes pacificus*. *J. Nat. Hist.*, 1–8. doi:10.1080/00222933.2016.1244297.

Tercero, J. F., Rosas, C., Mascaro, M., Poot, G., Domingues, P., Noreña, E., et al. (2015). Effects of parental diets supplemented with different lipid sources on *Octopus maya* embryo and hatching quality. *Aquaculture* 448, 234–242. doi:10.1016/j.aquaculture.2015.05.023.

Tian, K., Lou, F., Gao, T., Zhou, Y., Miao, Z., and Han, Z. (2018). De novo assembly and annotation of the whole transcriptome of *Sepiella maindroni*. *Mar. Genomics* 38, 13–16. doi:10.1016/j.margen.2017.06.004.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., et al. (2012). Primer3-new capabilities and interfaces. *Nucleic Acids Res.* 40, 1–12. doi:10.1093/nar/gks596.

Van Heukelem, W.F. 1983. *Octopus maya* In: Boyle, P.R. (Ed.), Cephalopod Life Cycles. Vol.I. Academic Press, London, pp: 311-323.

Van Heukelem, W.F. 1977. Laboratory maintenance, breeding, rearing and biomedical research potential of the Yucatan octopus (*Octopus maya*). Lab. Anim. Sci. 27, 852 859.

Voight, J. R., and Feldheim, K. A. (2009). Microsatellite inheritance and multiple paternity in the deep-sea octopus *Graneledone boreopacifica* (Mollusca : Cephalopoda). *Invertebr. Biol.* 128, 26–30. doi:10.1111/j.1744-7410.2008.00152.x.

Voight, J.R. 2002. Morphometric analysis of male reproductive features of Octopodids (Mollusca: Cephalopoda). Biol. Bull. 202: 148-155.

Voss, G.L. y Solis-Ramírez, M.J. 1966. *Octopus maya*, a new species from the Bay of Campeche. Bulletin of Marine Science 16, 615-625.

Waiho, K., Fazhan, H., Shahreza, M. S., Moh, J. H. Z., Noorbaiduri, S., Wong, L. L., et al. (2017). Transcriptome analysis and differential gene expression on the testis of orange mud crab, *Scylla olivacea*, during sexual maturation. *PLoS One* 12, 1–29. doi:10.1371/journal.pone.0171095.

Wang, S., Zheng, H., Esaki, Y., Kelly, F., and Yan, W. (2006). Cullin3 Is a KLHL10-Interacting Protein Preferentially Expressed During Late Spermiogenesis. *Biol. Reprod.* 74, 102–108. doi:10.1095/biolreprod.105.045484.

Wang, W., Hui, J. H. L., Chan, T. F., and Chu, K. H. (2014). De Novo Transcriptome Sequencing of the Snail *Echinolittorina malaccana* : Identification of Genes Responsive to Thermal Stress and Development of Genetic Markers for Population Studies. *Mar. Biotechnol.* 16, 547–559. doi:10.1007/s10126-014-9573-0.

Wodinsky, J. 2008. Reversal and transfer of spermatophores by *Octopus vulgaris* and *O. hummelincki*. Mar. Biol. 155: 91-103.

Xu, B., Hao, Z., Jha, K. N., Zhang, Z., Urekar, C., Digilio, L., et al. (2008). Targeted deletion of Tssk1 and 2 causes male infertility due to haploinsufficiency. *Dev. Biol.* 319, 211–222. doi:10.1016/j.ydbio.2008.03.047.

Yan, W., Si, Y., Slaymaker, S., Li, J., Zheng, H., Young, D. L., et al. (2010). Zmynd15 encodes a histone deacetylase-dependent transcriptional repressor essential for spermiogenesis and male fertility. *J. Biol. Chem.* 285, 31418–31426. doi:10.1074/jbc.M110.116418.

Yatsenko, A. N., Iwamori, N., Iwamori, T., and Matzuk, M. M. (2010). The power of mouse genetics to study spermatogenesis. *J. Androl.* 31, 34–44. doi:10.2164/jandrol.109.008227.

Ylitalo-ward, H. A. (2014). Life history, mating behaviour and multiple paternity in *Octopus oliveri* (Berry, 1914) (Cephalopoda: Octopodidae).

Yoshida, M. A., Yamada, L., Ochi, H., Iwata, Y., Tamura-Nakano, M., Sawada, H., et al. (2014). Integrative omics analysis reveals differentially distributed proteins in dimorphic euspermatozoa of the squid, *Loligo bleekeri*. *Biochem. Biophys. Res. Commun.* 450, 1218–1224. doi:10.1016/j.bbrc.2014.04.076.

Zavala-Hidalgo, J., Gallegos-García, A., Martínez-López, B., Morey, S. L., and O'Brien, J. J. (2006). Seasonal upwelling on the Western and Southern Shelves of the Gulf of Mexico. *Ocean Dyn.* 56, 333–338. doi:10.1007/s10236-006-0072-3.

Zavala-Hidalgo, J., Morey, S. L., and O'Brien, J. J. (2003). Seasonal circulation on the western shelf of the Gulf of Mexico using a high-resolution numerical model. *J. Geophys. Res.* 108, 1–19. doi:10.1029/2003JC001879.

Zhang, H., Su, D., Yang, Y., Zhang, W., Liu, Y., Bai, G., et al. (2010). Some single-nucleotide polymorphisms of the TSSK2 gene may be associated with human spermatogenesis impairment. *J. Androl.* 31, 388–392. doi:10.2164/jandrol.109.008466.

Zhang, X., Mao, Y., Huang, Z., Qu, M., Chen, J., Ding, S., et al. (2012). Transcriptome analysis of the *Octopus vulgaris* central nervous system. *PLoS One* 7, e40320. doi:10.1371/journal.pone.0040320.

Zuñiga, R. 0., Olivares, P.A. and Ossandón, R. L.. (1995). Influencia de la luz en la maduración sexual de hembras de *Octopus mimus*. Estud. Oceanol., 14:75-76.

Supplementary Material

Supplementary material 1: Chapter 2

Table 9. Allele frequencies for each microsatellite locus of *O. maya* males.

	Omy2-0		Omy2-07		Omy4-01		Omy4-11		Omy4-18	
Breeders	**Allele**	**Freq.**	**Allele**	**Freq.**	**Allele**	**Freq.**	**Allele**	**Freq.**	**Allele**	**Freq.**
	211	0.06522	193	0.15217	211	0.06522	236	0.04348	156	0.15217
	215	0.02174	196	0.06522	215	0.02174	240	0.08696	160	0.67391
	217	0.17391	199	0.73913	219	0.32609	242	0.43478	164	0.15217
	219	0.02174	202	0.04348	223	0.3913	244	0.02174	176	0.02174
	221	0.26087			227	0.04348	246	0.3913		
	223	0.15217			231	0.04348	250	0.02174		
	225	0.19565			243	0.06522				
	227	0.08696			247	0.02174				
	233	0.02174			259	0.02174				
Offsprings	211	0.0123	168	0.00205	143	0.00205	236	0.01025	154	0.00615
	217	0.19262	193	0.15164	168	0.00615	240	0.07787	156	0.06557
	219	0.03689	194	0.00205	211	0.07377	242	0.46311	160	0.73975
	221	0.27254	196	0.05533	215	0.00205	244	0.04508	162	0.00205
	223	0.21721	199	0.67418	219	0.38934	246	0.36066	164	0.16189
	225	0.17008	202	0.04098	223	0.3627	250	0.04303	176	0.01844
	227	0.02049	208	0.04303	227	0.02664			180	0.00615
	229	0.0041	211	0.0041	231	0.06148				
	231	0.00615	217	0.00615	232	0.00205				
	233	0.05123	220	0.0123	239	0.01639				
	235	0.00205	223	0.00615	243	0.05328				
	253	0.01434	225	0.00205	259	0.0041				

Freq. – Allele frequencies obtained with ARLEQUIN software.

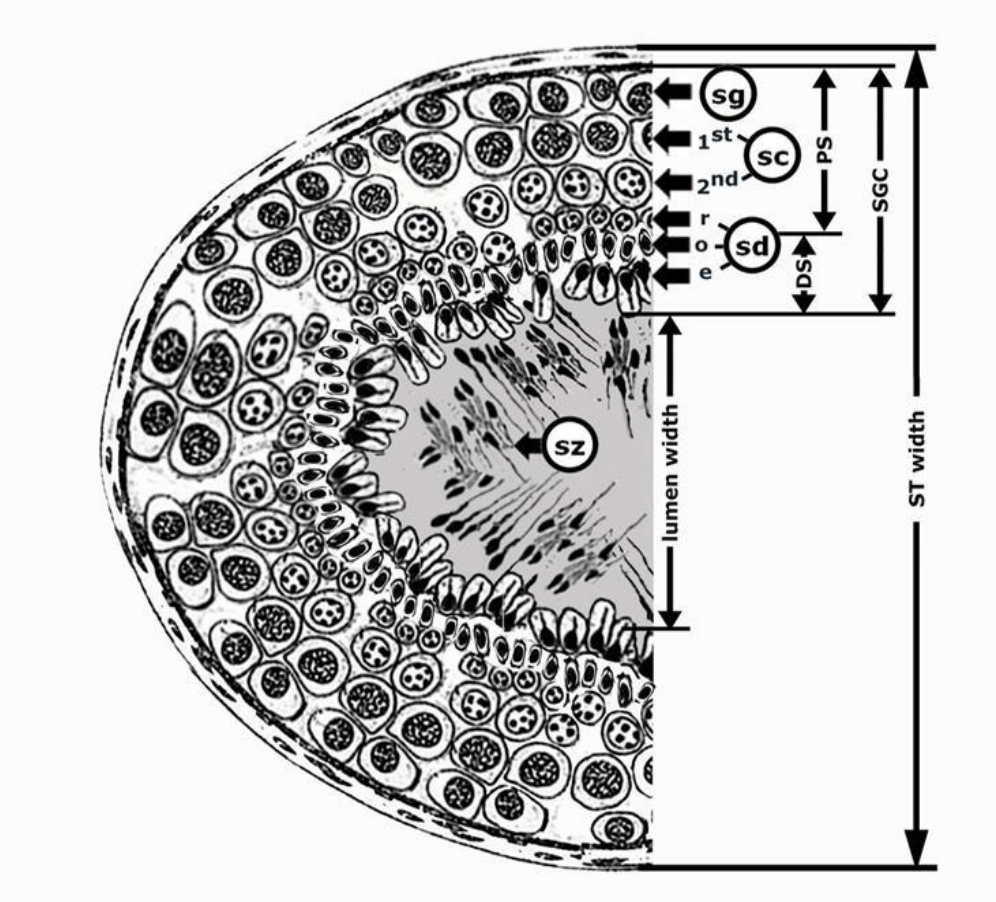

Figure 19. Schematic drawing of one seminiferous tubule (ST) in longitudinal section showing measured widths and heights. DS-differentiation stratum, PS- proliferative stratum, SGC- strata of germ cells (SGC = PS + DS), sg- spermatogonia, sc- spermatocytes, sd- spermatids (round, ovoid and elongated), sz- spermatozoa. The grey shadow represents the lumen where sperm is free (spermiation).

Supplementary material 2: Chapter 3

Table 10. Designed primers for selected DEG's used in qRT-PCR assays. GC- Guanine-Cytosine percentage; T - annealing temperature; e - Primer efficiency.

Gen	RNA-seq Transcript		Primer Sequence (5'→3')	Position	Length	Fragment length (bp)	GC%	T (°C)	*E*
NFKB2	TRINITY_DN11092_c0_g1_i1	F	CTTCATGGCCAGTAGTACTTGC	2435-2456	22	153	50.0	60	1.992
		R	AGAAAGAAGGCCCTGTCAAAAC	2566-2587	22		45.5		
ZMYND15	TRINITY_DN17130_c0_g1_i2	F	ATAATGAACTCCTCTCCGGGAC	36-57	22	125	50.0	56	1.903
		R	ACATTCTCAACACGTCCGTTAC	139-160	22		45.5		
TDRD1	TRINITY_DN9963_c0_g1_i1	F	GTGAGGGTTGTCATCAGAATCG	494-515	22	176	50.0	56	1.942
		R	GTTTGATTGATTTGGGTCACGC	648-669	22		45.5		
HTT	TRINITY_DN2386_c0_g1_i1	F	CGATTTCTTCCCACCTCAAGAC	2652-2673	22	160	50.0	62	1.905
		R	CAACATAACCCAATCACGGACA	2790-2811	22		45.5		
MIF	TRINITY_DN13245_c0_g1_i1	F	GCCAATCTGCTTCGACATCATT	314-335	22	165	45.5	60	1.99
		R	GCAGTTCTCTCCCTCGATTTTG	457-478	22		50.0		
GPX4	TRINITY_DN26602_c0_g1_i1	F	TTCCTCCGGTTCTGTTTGATTG	29-50	22	155	45.5	60	1.909
		R	CCATCAATAGTCGTGGCAGAAA	162-183	22		45.5		
HSPA9	TRINITY_DN33756_c0_g1_i1	F	TTTATGATCTTGGCGGTGGAAC	838-859	22	173	45.5	62	1.969
		R	GGACACATCTACTCCTTGGTCT	989-1010	22		50.0		
CASP7	TRINITY_DN7707_c0_g1_i1	F	ATTTGGCTGAGGTTCACGATTT	672-693	22	151	40.9	60	1.991
		R	TCCTCGTTCGCATTAACTTTCC	801-822	22		45.5		
KLHL10	TRINITY_DN16585_c0_g1_i1	F	TCTTCCCCTCCTCTTTGCTATC	2964-2985	22	124	50.0	60	1.939
		R	CAAATATTCCAAGGTCCCGGAC	3066-3087	22		50.0		
TSSK2	TRINITY_DN17275_c0_g1_i1	F	GCGCCGTACATTGTTCAAATAC	3020-3041	22	158	45.5	60	1.968
		R	CGCCTGCCATATTGTGTAGAAT	3156-3177	22		45.5		
RABL2A	TRINITY_DN7210_c0_g1_i1	F	TGACCTGCAGTATCCCAGAAAT	754-775	22	101	45.5	60	1.929

Gen	RNA-seq Transcript		Primer Sequence (5'→3')	Position	Length	Fragment length (bp)	GC%	T (°C)	*E*
		R	AAACCACAACAATCATCCACGT	833-854	22		40.9		
CHD5	TRINITY_DN16555_c1_g1_i2	F	CAAACCTCTGCCACCTTTGTTA	1937-1958	22	153	45.5	60	1.959
		R	AAATCTCGTACCAGCCATTGTG	2068-2089	22		45.5		
DNAJB13	TRINITY_DN3007_c0_g1_i1	F	CATTGAAAGACCATCCACACGA	744-765	22	191	45.5	60	1.98
		R	TCCTTCTCCTGGCACTACTTTT	913-934	22		45.5		
TUFM	TRINITY_DN35138_c0_g1_i1	F	TTTTCCCCATAGATTCGGCTGT	724-745	22	117	45.5	60	2.026
		R	AAGCACCGTGTCCCAGTATATC	819-840	22		50.0		
TUBGCP3	TRINITY_DN14290_c0_g1_i1	F	TTGATGGAGAGCAGCACAGAAA	2043-2064	22	151	45.5	60	1.963
		R	GATCGGAATAGGGAGAGCAGTG	2172-2193	22		54.6		

Table 11. Search for key genes in stress response in testis of *O. maya* exposed to thermal stress and reproductive condition. OSR – Oxidative stress response, PDC – Programmed death cell, RAP – Regulation of apoptotic process, RSR – Regulation of stress response, ROS – Response to oxidative stress, SR – stress response, IR – Inflammatory response. Asterisk indicates the treatment where the genes were significantly up-regulated. Proteins in bold indicate the good candidates to qPCR analysis.

Contig ID	UniProt ID	Protein Name	24POST	30PRE	30POST	Biological process	E-value
TRINITY_DN17317_c0_g1_i	STK24_HUMAN	Serine/threonine-protein kinase 24			*	OSR	6.02E-150
TRINITY_DN7707_c0_g1_i1	CASP7_HUMAN	**Caspase-7**			*	PDC	8.99E-25
TRINITY_DN33756_c0_g1_i	GRP75_PONAB	**Stress-70 protein**			*	RAP	0.00E+00
TRINITY_DN1469_c0_g2_i1	ABR_MOUSE	Active breakpoint cluster region-related protein		*	*	RSR	2.11E-28
TRINITY_DN15474_c0_g1_i	BABA1_DANRE	BRISC and BRCA1-A complex member 1		*	*	RSR	4.81E-49
TRINITY_DN12962_c0_g1_i	CO3_HUMAN	Complement C3		*	*	RSR	4.98E-81
TRINITY_DN641_c0_g1_i1	PGBD3_HUMAN	PiggyBac transposable element-derived protein 3		*		RSR	1.76E-29
TRINITY_DN16710_c0_g1_i	PPM1B_RAT	Protein phosphatase 1B		*	*	RSR	6.94E-177
TRINITY_DN17962_c9_g2_i	SETMR_HUMAN	Histone-lysine N-methyltransferase SETMAR		*	*	RSR	1.11E-40
TRINITY_DN7225_c0_g2_i1	SH3R1_XENTR	E3 ubiquitin-protein ligase SH3RF1			*	RSR	1.54E-16
TRINITY_DN17905_c8_g1_i	UBCD6_DROME	Ubiquitin-conjugating enzyme E2-17 kDa			*	RSR	4.42E-91
TRINITY_DN15904_c0_g1_i	CSK_CHICK	Tyrosine-protein kinase CSK			*	ROS	0.00E+00
TRINITY_DN26602_c0_g1_i	GPX4_BOVIN	**Phospholipid hydroperoxide glutathione**			*	ROS	6.74E-72
TRINITY_DN49654_c0_g1_i	KPYM_HUMAN	Pyruvate kinase PKM			*	ROS	7.93E-117

Contig ID	UniProt ID	Protein Name	24POST	30PRE	30POST	Biological process	E-value
TRINITY_DN10096_c0_g1_i	SODM_RAT	Superoxide dismutase [Mn]			*	ROS	7.07E-102
TRINITY_DN11092_c0_g1_i	NFKB2_MOUSE	**Nuclear factor NF-kappa-B p100 subunit**		*	*	SR/IR	2.70E-100
TRINITY_DN14073_c0_g1_i	C1QBP_BOVIN	Complement component 1 Q subcomponent-			*	SR	1.65E-24
TRINITY_DN12030_c0_g1_i	CRIP1_HUMAN	Cysteine-rich protein 1			*	SR	5.59E-29
TRINITY_DN13057_c0_g1_i	DDX58_MOUSE	Probable ATP-dependent RNA helicase DDX58			*	SR	3.25E-72
TRINITY_DN11341_c0_g1_i	FLNA_HUMAN	Filamin-A			*	SR	0.00E+00
TRINITY_DN50655_c0_g1_i	HSP7C_RAT	Heat shock cognate 71 kDa protein			*	SR	0.00E+00
TRINITY_DN42859_c0_g1_i	ITA9_MOUSE	Integrin alpha-9			*	SR	3.83E-49
TRINITY_DN26506_c0_g1_i	MALT1_HUMAN	Mucosa-associated lymphoid tissue lymphoma			*	SR	4.19E-50
TRINITY_DN13245_c0_g1_i	MIF_PIG	**Macrophage migration inhibitory factor**			*	SR/IR	1.18E-29
TRINITY_DN16861_c0_g1_i	NAC3_HUMAN	Sodium/calcium exchanger 3			*	SR	1.55E-66
TRINITY_DN9614_c0_g1_i1	PDCD6_HUMAN	Programmed cell death protein 6			*	SR	9.47E-75
TRINITY_DN8637_c0_g1_i1	PLCG1_MOUSE	1-phosphatidylinositol 4			*	SR	1.70E-115
TRINITY_DN18002_c2_g2_i	RIPK1_HUMAN	Receptor-interacting serine/threonine-protein		*		SR	1.53E-06

Table 12. Search for key genes in the reproductive process and male fertility in testis of *O. maya* exposed to thermal stress and reproductive condition. GG – Gamete generation, SG – Spermatogenesis, SpG – Spermiogenesis. Asterisk indicates the treatment where the genes were significantly up-regulated. Proteins in bold indicate the good candidates to qPCR analysis.

Contig ID	UniProt ID	Protein Name	24POST	30PRE	30POST	Biological process	E-value
TRINITY_DN12132_c0_g1_i1	KDM1B_MOUSE	Lysine-specific histone demethylase 1B			*	GG	2.50E-151
TRINITY_DN3082_c0_g2_i1	MMP19_HUMAN	Matrix metalloproteinase-19			*	GG	9.53E-49
TRINITY_DN3007_c0_g1_i1	DJB13_MOUSE	**DnaJ homolog subfamily B member 13**			*	SG	1.23E-122
TRINITY_DN26459_c0_g1_i1	AGM1_HUMAN	Phosphoacetylglucosamine mutase			*	SG	0.00E+00
TRINITY_DN7460_c0_g2_i1	ASPM_FELCA	Abnormal spindle-like microcephaly-associated			*	SG	2.56E-77
TRINITY_DN16555_c1_g1_i2	CHD5_MOUSE	**Chromodomain-helicase-DNA-binding protein 5**			*	SG	0.00E+00
TRINITY_DN2386_c0_g1_i1	HD_MOUSE	**Huntingtin**			*	SG	0.00E+00
TRINITY_DN17881_c0_g1_i3	PSME4_BOVIN	Proteasome activator complex subunit 4	*	*		SG	0.00E+00
TRINITY_DN7210_c0_g1_i1	RBL2A_PONAB	**Rab-like protein 2A**		*		SG	5.95E-82
TRINITY_DN9963_c0_g1_i1	TDRD1_ORYLA	**Tudor domain-containing protein 1**		*	*	SG	2.77E-13
TRINITY_DN17275_c0_g1_i1	TSSK2_MOUSE	**Testis-specific serine/threonine-protein kinase 2**	*	*		SG	6.82E-47
TRINITY_DN17766_c0_g1_i1	ZAN_PIG	Zonadhesin		*		SG	3.39E-50
TRINITY_DN27478_c0_g1_i1	ITB1_SHEEP	Integrin beta-1			*	SG	2.29E-128
TRINITY_DN16585_c0_g1_i1	KLH10_HUMAN	**Kelch-like protein 10**		*	*	SpG	2.68E-142
TRINITY_DN17130_c0_g1_i2	ZMY15_HUMAN	**Zinc finger MYND domain-containing protein 15**		*		SpG	3.03E-53

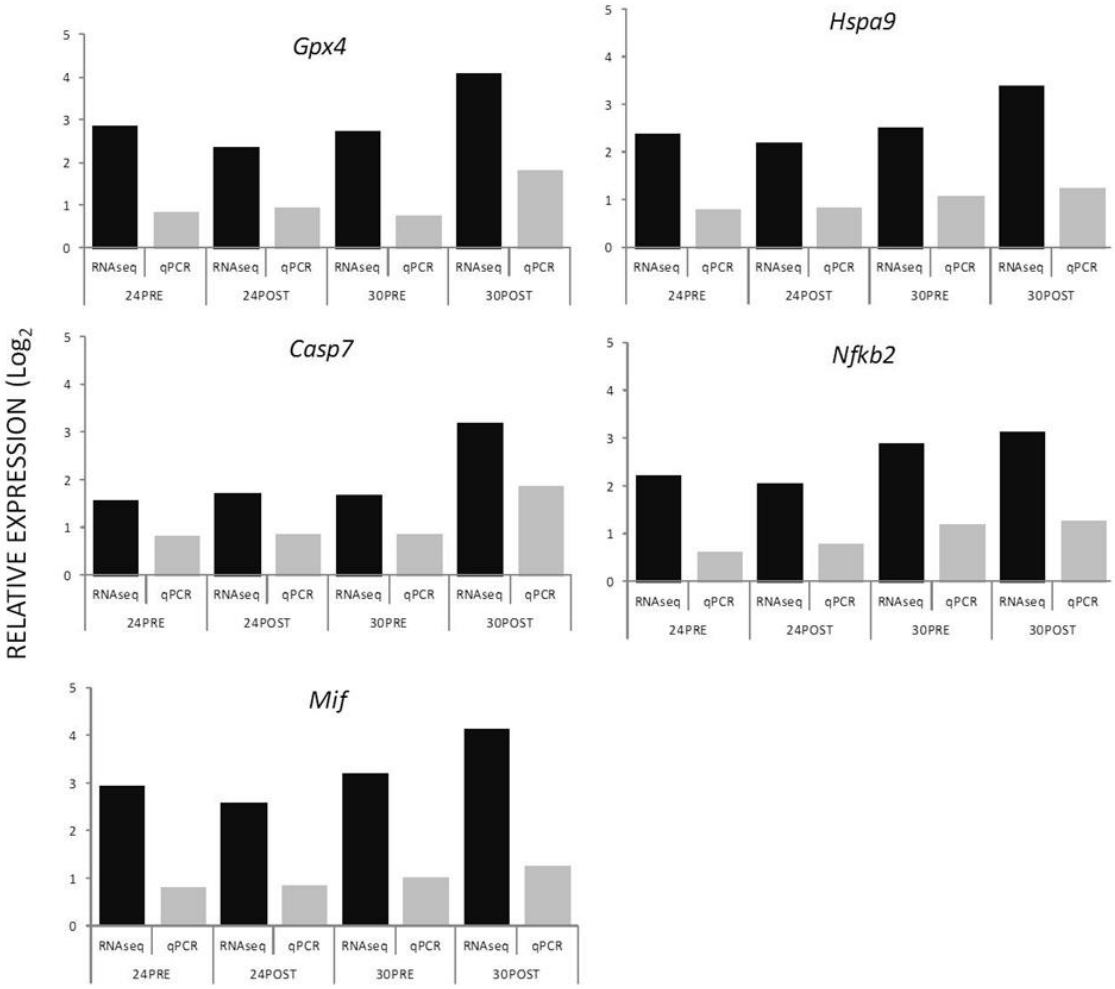

Figure 20. RNA-Seq and qPCR $\log_2$ transformed expression for transcripts associated with thermal stress response.

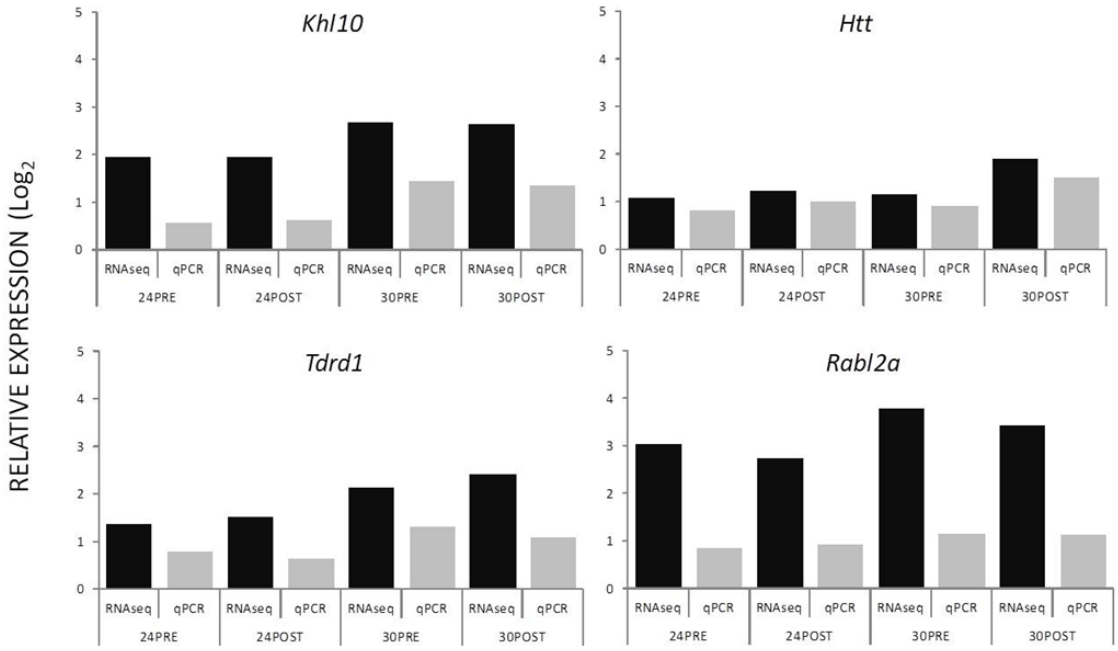

Figure 21. RNA-Seq and qPCR log_2 transformed expression for transcripts involved in the reproductive process.