been completely sequenced to date, estimates are that the American lobster (*Homarus americanus*) genome is about 4.40 pg/4.3 Gbp or greater (see Animal Genome Size Database website and Gloucester Marine Genomics Institute website, American Lobster Genome, on the internet).

[007] The lack of information regarding shrimp, prawn, crab, crayfish, and/or lobster is arguably largely due to challenges in cell culture. For example, shrimp/prawn cell lines have only been established from the lymphoid organ and ovaries (Tapay *et al*. (1995) Proc Soc Exp Biol Med 209(1):73-8; Hsu *et al*. (1995) Aquaculture 136:43-55; US patent number 6143547; George and Dhar (2010) In Vitro Cell Dev Biol Anim 46(9):801-10; Ma *et al*. (2017) Reviews in Aquaculture 9: 88-98). In addition, shrimp/prawn primary muscle cultures have lasted only up to 12 days (George and Dhar, 2010) and no known primary cultures or cell lines have been described for fat cells.

[008] Furthermore, attempts to culture fat cells from shrimp, prawn, crab, crayfish, and/or lobster have not been described.

[009] The availability of immortalized cell lines from shrimp, prawn, crab, crayfish, and/or lobster would allow cellular agriculture companies to progress to the next steps of cellular agriculture (Rubio *et al*., (2019) Front. Sustain. Food Syst. doi: 10.3389/fsufs.2019.00043). Rather than the time consuming process of isolating primary cells, food regulatory and research groups would then have a ready source of cell lines. This would facilitate understanding shrimp, prawn, crab, crayfish, and/or lobster viral diseases, testing for the presence of those viruses, and developing treatments (Ma *et al*. (2017) Reviews in Aquaculture 9: 88-98). Consequently, there remains a need for immortalized muscle and fat cell lines from shrimp, prawn, crab, crayfish, and/or lobster. The disclosure presented herein addresses this need.

SUMMARY

[009a] In one aspect, the present disclosure refers to a crustacean muscle stem cell line obtained from isolated primary muscle stem cells, wherein the isolated primary muscle stem cells are selected from the group consisting of shrimp, prawn, lobster, crab, and crayfish.

[009b] In another aspect, the present disclosure refers to a method for producing a shrimp and/or prawn muscle stem cell line according to claim 1 comprising (a) isolating shrimp and/or prawn muscle; (b) sterilizing the isolated muscle and removing fat and epidermal tissue comprised in the muscle; (c) mincing the isolated muscle after step (b) to obtain minced muscle tissue sections, and placing the minced muscle tissue sections into a cell culture with a cell culture media, wherein the minced muscle tissue sections comprise cells; (d) incubating the cell culture of step (c) at 28°C without CO_2 for 3 to 7 days; (e) removing the minced muscle tissue sections from the cell culture, wherein the cells comprised in the muscle tissue sections remain in the cell culture; (f) adding fresh media to the cell culture and incubating the cells in the cell culture at 28°C without CO_2 for 3 to 7 days; and (g) repeating step (f) when cells number reaches about 8 million in 10 ml of media, wherein step (f) is repeated at least 10 times.

[009c] In another aspect, the present disclosure refers to cell cultures comprising the crustacean muscle stem cell lines as described herein.

[009d] In another aspect, the present disclosure refers to a cell culture comprising the crustacean muscle stem cell line as described herein, wherein the crustacean muscle stem cell line is shrimp, and/or prawn muscle cells from a species belonging to the genus *Penaeus*, *Solenocera*, *Metapenaeus*, *Parapenaeus*, *Parapenaeopsis*, *Metapenaeopsis*, *Trachypenaeus*, *Protrachypene*, *Xiphopenaeus*, *Hymenopenaeus*, *Atypopenaeus*, *Eusicyonia*, *Sicyonia*, or *Litopenaeus*.

[009e] In yet another aspect, the present disclosure refers to a cell culture comprising the crustacean muscle stem cell line as described herein, wherein the crustacean muscle stem cell line is shrimp, and/or prawn muscle stem cells, wherein the shrimp, and/or prawn muscle stem cells are from *Penaeus monodon* and/or *Litopenaeus vannamei*.

[009f] In another aspect, the present disclosure refers to a method for producing a crab muscle stem cell line according to claim 1 comprising (a) isolating crab muscle from claws, legs, and

bodies of freshly sacrificed crabs; (b) sterilizing the isolated muscle and removing fat and epidermal tissue comprised in the muscle; (c) mincing the isolated muscle after step (b) to obtain minced muscle tissue sections, and placing the minced muscle tissue sections into a cell culture with a cell culture media, wherein the minced muscle tissue sections comprise muscle stem cells; (d) incubating the cell culture of step (c) at 20-24^{o}C without CO_2 for about 7 days; (e) removing the minced muscle tissue sections from the cell culture, wherein the muscle stem cells comprised in the muscle tissue sections remain in the cell culture; (f) adding fresh media to the cell culture and incubating the cells in the cell culture at 20-24^{o}C without CO_2; and (g) repeating step (f) when cells number reaches about 8 million in 10 ml of media, wherein step (f) is repeated at least 10 times.

[009g] In another aspect, the present disclosure refers to a method for producing a crayfish or lobster muscle stem cell line according to claim 1 comprising (a) isolating crayfish or lobster muscle from claws, legs, and tails of freshly sacrificed crayfish or lobster; (b) sterilizing the isolated muscle and removing fat and epidermal tissue comprised in the muscle; (c) mincing the isolated muscle after step (b) to obtain minced muscle tissue sections, and placing the minced muscle tissue sections into a cell culture with a cell culture media, wherein the minced muscle tissue sections comprise muscle stem cells; (d) incubating the cell culture of step (c) at 27^{o}C without CO_2 for about 7 days for crayfish or at saturated humidity at 5^{o}C for lobster; (e) removing the minced muscle tissue sections from the cell culture, wherein the muscle stem cells comprised in the muscle tissue sections remain in the cell culture; (f) adding fresh media to the cell culture and incubating the cells in the cell culture at 27^{o}C without CO_2 for crayfish or at saturated humidity at 5^{o}C for lobster; and (g) repeating step (f) when cells number reaches about 8 million in 10 ml of media, wherein step (f) is repeated at least 10 times.

[010] The present disclosure is directed to tissue engineered shrimp, prawn, crab, crayfish, and/or lobster meat products and methods for producing such products. In one example, the meat product comprises muscle cells that are grown *ex vivo* and/or *in vitro*. In a further example, the

[068] Culture media is changed and cells are washed 72 hours after plating as described in Bunnell *et al*. (Methods 2008; 45(2):115-120). To maintain cells afterwards, culture media is changed every second day until cells reach 80-90% confluence. The cells can be harvested or differentiated once they reach 80-90% confluence, as described in Bunnell *et al*. (Methods 2008; 45(2):115-120).

Example 6 – Isolation of primary muscle stem cells from crab, crayfish, and lobster

[069] Following sacrifice and cleaning of crabs or lobster on ice as described in [049] and [050], muscle of crab claws, legs and main body are isolated and processed as described in [051]-[053]. The crab muscle stem cells are cultured at 20-24°C as described in Sashikumar and Desai (Cytotechnology (2008) 56:161-169).

[070] For crayfish, and lobsters, muscle from the claws, legs and tail is isolated, processed and cultured as described in [051]-[053]. Crayfish muscle stem cells are cultured at 27°C as described in Neumann *et al*. (In Vivo (2000) 14(5): 691-8). Lobster muscle stem cells are cultured at saturated humidity at 5°C as described in Stepanyan *et al*. (Chemical Senses (2004) 29(3):179-187) or the temperature at which the lobsters were kept while alive.

CLAIMS

We claim:

1. A crustacean muscle stem cell line obtained from isolated primary muscle stem cells, wherein the isolated primary muscle stem cells are selected from the group consisting of shrimp, prawn, lobster, crab, and crayfish.

2. The crustacean muscle stem cell line according to claim 1, wherein the muscle stem cells differentiate into myofibers.

3. The crustacean muscle stem cell line of claim 1, wherein the isolated primary muscle stem cells are shrimp or lobster primary muscle stem cells.

4. A method for producing a shrimp and/or prawn muscle stem cell line according to claim 1 comprising

(a) isolating live adult or postlarval shrimp and/or prawns on ice for 10 minutes prior to sacrificing;

(b) rinsing the whole shrimp or prawns in 11% sodium hypochlorite for 10 seconds prior to dissecting the last body segment and tail

(c) sterilizing the dissected tails and attached segments in 2% potassium permanganate solution for 10 minutes, then rinsing once in phosphate buffered saline (PBS; 0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, 0.0018 M KH_2HPO_4, pH 7.4), then once in 2% penicillin-streptomycin filtered PBS and then holding in 70% ethanol for 5-10 minutes prior to washing in 5% or 10% penicillin-streptomycin filtered PBS for 5-10 minutes;

(d) (i) for adult muscle isolations, removing and reserving the tail and dissecting opaque muscle from the body segment and removing fat and epidermal tissue; or

(d) (ii) for postlarval muscle isolation, separating the head from the body, removing legs and mincing the body segments with an electrical mincer or handheld blender;

(e) manually mincing the adult or postlarval muscle into pieces approximately 1.0 mm in size prior to transfer into a 10 cm dish containing one of the following cell culture media:

i. Grace's insect media with 10% heat-inactivated fetal bovine serum and 5% penicillin-

streptomycin;

ii.DMEM high glucose with 10% heat-inactivated bovine serum and 5% penicillin-streptomycin;

iii. NUTRISTEM® MSC XF supplement mix: NUTRISTEM® MSC XF basal media with 5% penicillin-streptomycin;

iv. DMEM high glucose: HYCLONETM : MYOCULTTM SF expansion human 10x: 5% penicillin-streptomycin; or

v. HYCLONETM media for undifferentiated mesenchymal stem cells: HYCLONETM stem cell 10x Supplement: 5% penicillin-streptomycin,

and mincing again;

(f) for adult tissue, removing muscle from the reserved tail, mincing, transferring into the 10cm dish containing culture media and mincing again

(g) transferring the minced tissue and cell culture media to cell culture flasks and/or dishes and incubating at 28ºC without CO_2 for 3 to 7 days with fresh media added every 2-3 days;

(h) removing tissues by gravity separation or centrifugation for 10-30 seconds then splitting the remaining cell population by density,

adding fresh media to a final volume of 10-12 ml per T75 flask, and continuing incubation at 28ºC with fresh cell culture media added every 2-3 days;

(i) when cells number about 8 million in 10 ml of culture media,

again dividing said cells and diluting said cells at a 1:3 ratio and incubating under the same conditions until passage 2;

(j) centrifuging a portion of the cells for 5 minutes at 1000 x g, removing supernatant and resuspending the cells in cryotubes in 1ml of Bambanker freezing media prior to storage at -80°C; and

(k) culturing the remaining portion of cells in cell culture media until at least passage 4 with antibiotics present in the culture media reduced to 2% after passage 2 and further reduced to 0% after passage 4.

5. A cell culture comprising the crustacean muscle stem cell line according to claim 1.

6. A cell culture comprising the crustacean muscle stem cell line of claim 3.

7. A cell culture comprising the crustacean muscle stem cell line according to claim 4.

8. A method for producing a crab muscle stem cell line according to claim 1 comprising

(a) isolating crab muscle from claws, legs, and bodies of freshly sacrificed crabs;

(b) sterilizing the isolated muscle and removing fat and epidermal tissue comprised in the muscle;

(c) mincing the isolated muscle after step (b) to obtain minced muscle tissue sections, and placing the minced muscle tissue sections into a cell culture with a cell culture media, wherein the minced muscle tissue sections comprise muscle stem cells;

(d) incubating the cell culture of step (c) at 20-24^{o}C without CO_2 for about 7 days;

(e) removing the minced muscle tissue sections from the cell culture, wherein the muscle stem cells comprised in the muscle tissue sections remain in the cell culture;

(f) adding fresh media to the cell culture and incubating the cells in the cell culture at 20-24^{o}C without CO_2; and

(g) repeating step (f) when cells number reaches about 8 million in 10 ml of media,

wherein step (f) is repeated at least 10 times.

9. A method for producing a crayfish or lobster muscle stem cell line according to claim 1 comprising

(a) isolating crayfish or lobster muscle from claws, legs, and tails of freshly sacrificed crayfish or lobster;

(b) sterilizing the isolated muscle and removing fat and epidermal tissue comprised in the muscle;

(c) mincing the isolated muscle after step (b) to obtain minced muscle tissue sections, and placing the minced muscle tissue sections into a cell culture with a cell culture media, wherein the minced muscle tissue sections comprise muscle stem cells;

(d) incubating the cell culture of step (c) at 27^{o}C without CO_2 for about 7 days for crayfish or at saturated humidity at 5^{o}C for lobster;

(e) removing the minced muscle tissue sections from the cell culture, wherein the muscle stem cells comprised in the muscle tissue sections remain in the cell culture;

(f) adding fresh media to the cell culture and incubating the cells in the cell culture at 27°C without CO_2 for crayfish or at saturated humidity at 5°C for lobster; and

(g) repeating step (f) when cells number reaches about 8 million in 10 ml of media,

wherein step (f) is repeated at least 10 times.

10. A cell culture comprising the crustacean muscle stem cell line according to claim 1, wherein the crustacean muscle stem cell line is shrimp, and/or prawn muscle cells from a species belonging to the genus *Penaeus*, *Solenocera*, *Metapenaeus*, *Parapenaeus*, *Parapenaeopsis*, *Metapenaeopsis*, *Trachypenaeus*, *Protrachypene*, *Xiphopenaeus*, *Hymenopenaeus*, *Atypopenaeus*, *Eusicyonia*, *Sicyonia*, or *Litopenaeus*.

11. A cell culture comprising the crustacean muscle stem cell line according to claim 1, wherein the crustacean muscle stem cell line is shrimp, and/or prawn muscle stem cells, wherein the shrimp, and/or prawn muscle stem cells are from *Penaeus monodon* and/or *Litopenaeus vannamei*.